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The intracellular distribution of folate derivatives in pea (*Pisum sativum* L.) leaves

by

Sherwin Yum-Yat Chan

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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Edwin A. Cossins

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Dr. David J. Gifford

Arnost Horak

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Date: January 26, 1999.

To my family

ABSTRACT

Leaf extracts of pea *(Pisum sativum* L.) seedlings were examined for folate derivatives using *Lactobacillus rhamnosus*, *Pediococcus acidilactici*, and high performance liquid chromatography. Extracts of 14 day seedlings contained 0.17 ± 0.01 nmol folate mg^{-1} protein which was predominantly cytosolic and methylated. Extracts of gradient-purified mitochondria and chloroplasts contained 11.0 ± 2.3% and 8.4 ± 0.6% of cellular folate, respectively. Greater than 90% of mitochondrial folates (0.47 ± 0.11 nmol mg^{-1} protein) were formylated and unsubstituted. Methylated derivatives represented 30% of the chloroplastic pool (0.02 ± 0.001 nmol mg^{-1} protein).

Cellular folate increased 40% and was less γ-glutamyl conjugated when 9 day etiolated seedlings were greened for 48 hours. Greening also increased the mitochondrial folate pool 10-fold and was accompanied by a shift from methylated derivatives to formylated and unsubstituted folates. Changes in the folate pool of greening tissues are discussed in relation to the metabolism of glycine and serine that accompanies the onset of photorespiration.

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TABLE OF CONTENTS

1. INTRODUCTION 1

1.1. The Biochemistry of Folates 4

1.1.1. The chemical nature of naturally occurring folates 4

1.1.2. The biosynthesis of tetrahydrofolate 9

1.2. Pathways of One-carbon Metabolism 16

1.2.1. The glycine cleavage system 16

1.2.2. Folate-dependent serine metabolism 19

1.2.3. The interconversion of one-carbon units 22

1.2.3.1. The interconversion of 5-HCO- and 5,10-CH^{+}-$H_4PteGlu_n$ 22

1.2.3.2. The catabolism of 10-HCO-$H_4PteGlu_n$ 23

1.2.3.3. C_1-THF synthase activities of higher plants 24

1.2.4. Purine biosynthesis 29

1.2.5. Thymidylate biosynthesis 30

1.2.6. Methionine biosynthesis 31

1.2.7. Initiation of protein synthesis 33

1.3. Compartmentation of Folate Metabolism 33

1.3.1. Compartmentation of folate metabolism in higher plants 38

1.3.2. The present study 39

2. MATERIALS AND METHODS 41

2.1. Materials 41

2.2. Plant Material 42

2.3. Organelle Isolation and Extraction of Folates 42

2.3.1. Whole-leaf extracts 42

2.3.2. Cytosolic and mitochondrial extracts 43

2.3.3. Chloroplastic extracts 44

2.3.4. Assay of enzyme activities 44

2.4. Enzymatic Hydrolysis of Pteroylpolyglutamates 45

2.5. Microbiological Assay of Folate Derivatives 46

2.5.1. Glycerol-cryoprotected bacteria 46

2.5.2. Maintenance of microorganisms 46

2.5.3. Assay procedure 47

2.6. Separation of Pteroylmonoglutamate Derivatives by HPLC 49

3. RESULTS 51

3.1. γ-Glutamyl Hydrolase Treatment of Folate Samples 51

3.2. Release of Folates from Mitochondria and Chloroplasts 52

3.3. The Assay of Folate Derivatives 54

3.4. The Separation of Pteroylmonoglutamate Derivatives 56

3.5. The Folate Content of Pea Leaves 59

3.5.1. Folate content as a function of leaf age 59

3.5.2. Intracellular distribution of folate in 14 d pea leaves 64

3.6. The Effect of Greening on Folate Pools 69

3.6.1. Folate content of etiolated and greening pea leaves 69

3.6.2. Changes in the intracellular distribution of folate during greening 75

4. DISCUSSION 80

4.1. Evaluation of the Methods Employed in the Present Study 80

4.1.1. Microbiological assay of folates in microtiter plates 80

4.1.2. Hydrolysis of pteroylpolyglutamates 81

4.1.3. HPLC separation of pteroylmonoglutamates 84

4.2. The Folate Content of Pea Leaves 84

4.2.1. Folate content as a function of leaf age 84

4.2.2. Intracellular distribution of folate in 14 d pea leaves 86

4.3. The Effect of Greening on Folate Pools 90

4.3.1. Folate content of etiolated and greening pea leaves 90

4.3.2. Changes in the intracellular distribution of folate during greening 92

4.4. Conclusions 94

5. BIBLIOGRAPHY 96

LIST OF TABLES

Table 1. Responses of *L. rhamnosus, P. acidilactici, and E. hirae* to Various Pteroylglutamates. 8

Table 2. Solubilization of Pteroylglutamate Derivatives from Isolated Mitochondria. 55

Table 3. The Folate Content of 9 to 14 d Pea Leaves. 62

Table 4. Proportion of Total Folate Detected Before GGH Treatment in 9 to 14 d Pea Leaves. 63

Table 5. The Intracellular Distribution of Folate Derivatives in Leaves of 14 d Pea Seedlings. 65

Table 6. The Biochemical Purity of Subcellular Fractions Collected During Mitochondrial Isolation. 66

Table 7. The Intracellular Distribution of Folate Derivatives in Leaves of 9 d Etiolated Pea Seedlings. 76

Table 8. The Intracellular Distribution of Folate Derivatives in Leaves of 11 d Etiolated Pea Seedlings. 77

Table 9. The Intracellular Distribution of Folate Derivatives in Leaves of 9 d Etiolated Pea Seedlings Treated with 48 h of Light. 78

LIST OF FIGURES

Figure 1. Major Folate-dependent Pathways of One-carbon Metabolism. 2

Figure 2. Structure of: A. Pteroylglutamate and
B. Tetrahydropteroylpolyglutamate. 5

Figure 3. Biosynthesis of Tetrahydropteroylpolyglutamate from Pterin
Precursors. 10

Figure 4. Compartmentation of Folate-mediated One-carbon Metabolism. 34

Figure 5. The Hydrolysis of Folylpolyglutamates as a Function of
Incubation Time. 53

Figure 6. The Microbiological Assay of Folate Using 96-Well Microtiter Plates. 57

Figure 7. Standard Curve for the Microbiological Assay of Folate. 58

Figure 8. The Separation of Folate Derivatives by HPLC and *L. rhamnosus*
Assay. 60

Figure 9. Effect of Light on 9 d Etiolated Pea Seedlings. 70

Figure 10. Folate Content of Etiolated and Light-treated Pea Seedlings. 71

Figure 11. Degree of Conjugation of Folate Derivatives in Greening Experiments. 73

Figure 12. The Distribution of Individual Folate Derivatives in
Whole-leaf Extracts. 74

Figure 13. The pH-dependent Interconversions of Formyl-substituted Folates. 83

Figure 14. A Futile Cycle Involving SHMT and 5,10-CH^+-$H_4PteGlu_n$
Synthetase. 88

LIST OF ABBREVIATIONS

The abbreviations for folate derivatives are those suggested by the IUPAC-IUB Commission, as summarized by Blakley and Benkovic (1984).

5-CH_3-H_4PteGlu_n	5-methyltetrahydropteroylpolyglutamate
5-HCO-H_4PteGlu_n	5-formyltetrahydropteroylpolyglutamate
10-HCO-H_4PteGlu_n	10-formyltetrahydropteroylpolyglutamate
5,10-CH^+-H_4PteGlu_n	5,10-methenyltetrahydropteroylpolyglutamate
5,10-CH_2-H_4PteGlu_n	5,10-methylenetetrahydropteroylpolyglutamate
A	absorbance
Ab	antibody
ADP	adenosine-5'-diphosphate
AICAR	aminoimidazole carboxamide ribonucleotide
AIR	aminoimidazole ribonucleotide
ATCC	American Type Culture Collection
ATP	adenosine-5'-triphosphate
BSA	bovine serum albumin
C_1	one-carbon
C_1-THF synthase	trifunctional C_1-tetrahydrofolate synthase
CHES	2-(N-cyclohexylamino) ethanesulfonic acid
CHO	Chinese hamster ovary
DEAE	diethylaminoethyl

DHFR	dihydrofolate reductase
DHFS	dihydrofolate synthetase
DHPS	dihydropteroate synthase
DHY-CYC	5,10-methylenetetrahydrofolate dehydrogenase-5,10-methenyltetrahydrofolate cyclohydrolase bifunctional protein
DNA	deoxyribonucleic acid
dTMP	deoxythymidine monophosphate
dUMP	deoxyuridine monophosphate
fMet-tRNA	formylmethionyl-transfer ribonucleic acid
FPGS	folylpolyglutamate synthetase
GAR	glycinamide ribonucleotide
GDC	glycine decarboxylase
GGH	gamma-glutamyl hydrolase
GTP	guanosine-5'-triphosphate
H_2Pte	dihydropteroate
$H_2PteGlu$	7,8-dihydropteroylglutamate; dihydrofolate
$H_4PteGlu$	5,6,7,8-tetrahydropteroylglutamate; tetrahydrofolate
$H_4PteGlu_n$	5,6,7,8-tetrahydropteroylpoly-γ-glutamate, with *n* L-glutamate moieties

HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)
HPLC	high performance liquid chromatography
HPPK	hydroxymethyl dihydropterin pyrophosphokinase
MTX	4-amino-10-methylpteroylglutamate; methotrexate
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
p-ABA	*p*-aminobenzoic acid
P_i	inorganic orthophosphate
PteGlu	pteroylglutamic acid
R.H.	relative humidity
RNA	ribonucleic acid
SAM	*S*-adenosylmethionine
SEM	standard error of the mean
SHMT	serine hydroxymethyltransferase
SYN	10-formyltetrahydrofolate synthetase
TBAOH	tetrabutylammonium hydroxide
TBAP	tetrabutylammonium phosphate
THFA	tetrahydrofolic acid
TS	thymidylate synthase

INTRODUCTION

Folate derivatives, which are present in virtually every known organism and cell type, mediate one-carbon (C_1) transfers that play essential roles in several key cellular processes. Folate-dependent one-carbon metabolism is necessary for the syntheses of purines, thymidylate, formylmethionyl-tRNA (fMet-tRNA), glycine, serine, and methionine (Blakley, 1969; MacKenzie, 1984; Matthews, 1984; Rowe, 1984; Santi and Danenberg, 1984; Shane and Stokstad, 1984; Staben and Rabinowitz, 1984). One-carbon units, ranging in oxidation state from formyl to methyl (Cossins, 1984; Shane, 1989), are associated with tetrahydropteroylpolyglutamates ($H_4PteGlu_n$) and these derivatives actively participate in folate-dependent pathways (Figure 1). Furthermore, highly methylated plant compounds such as lignin, pectin, chlorophyll, anthocyanins, alkaloids, and flavonoids, are formed by transmethylation reactions with *S*-adenosylmethionine (SAM). This latter compound is synthesized indirectly by a folate-dependent pathway.

Ever since the discovery of pteroylglutamates (PteGlu), there has been intense research to elucidate their metabolic roles. Recently, interest in one-carbon metabolism has increased in light of its potential role in preventive medicine. While all organisms require a source of folate, not all are able to synthesize this molecule *de novo*. For mammals, folate is one of the ten essential vitamins that must be provided in the diet or by intestinal microorganisms (Herbert, 1990; Gregory, 1997). With

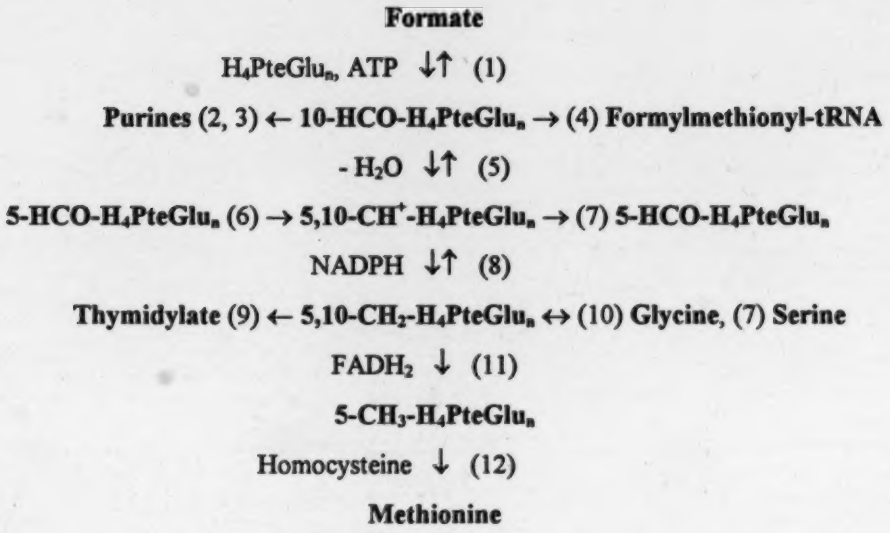

Formate
$H_4PteGlu_n$, ATP ↓↑ (1)
Purines (2, 3) ← 10-HCO-$H_4PteGlu_n$ → (4) Formylmethionyl-tRNA
- H_2O ↓↑ (5)
5-HCO-$H_4PteGlu_n$ (6) → 5,10-CH^+-$H_4PteGlu_n$ → (7) 5-HCO-$H_4PteGlu_n$
NADPH ↓↑ (8)
Thymidylate (9) ← 5,10-CH_2-$H_4PteGlu_n$ ↔ (10) Glycine, (7) Serine
$FADH_2$ ↓ (11)
5-CH_3-$H_4PteGlu_n$
Homocysteine ↓ (12)
Methionine

Figure 1. *Major Folate-dependent Pathways of One-carbon Metabolism.*

Enzymes catalyzing individual reactions are: 1. 10-formyltetrahydrofolate synthetase (SYN, EC 6.3.4.3), 2. glycinamide ribonucleotide (GAR) transformylase (EC 2.1.2.2), 3. aminoimidazole carboxamide ribonucleotide (AICAR) transformylase (EC 6.3.2.6), 4. methionyl-tRNA transformylase (EC 2.1.2.9), 5. 5,10-methenyltetrahydrofolate cyclohydrolase (CYC, EC 3.5.4.9), 6. 5,10-methenyltetrahydrofolate synthetase (EC 6.3.3.2), 7. serine hydroxymethyltransferase (SHMT, EC 2.1.2.1), 8. 5,10-methylenetetrahydrofolate dehydrogenase (DHY, EC 1.5.1.5), 9. thymidylate synthase (TS, EC 2.1.1.45), 10. glycine decarboxylase (GDC, EC 2.1.2.10), 11. 5,10-methylenetetrahydrofolate reductase (EC 1.5.1.20), and 12. methionine synthase (EC 2.1.1.13).

folate deficiency being implicated in the etiology of cardiovascular disease (Hopkins *et al*, 1995; Ward *et al*, 1997), megaloblastic anemia (Hoffbrand and Jackson, 1993; Campbell, 1995), neuropsychiatric disorders (Bottiglieri, 1996; Alpert and Fava, 1997), pregnancy complications including neural tube defects and abortion (Steegers-Theunissen, 1995; Pietrzik and Thorand, 1997), and cancer (Jennings, 1995; Mason and Levesque, 1996), the study of folate metabolism has intensified. Along with this heightened interest, folate investigators have witnessed an impressive development of new and improved methods for folate isolation and characterization. Important advances have also been made by the cloning, sequencing, and site-directed mutagenesis of genes that encode for major enzymes of folate metabolism. Of clinical interest, folate antagonists have found widespread use as antibacterial, antiprotozoal, antineoplastic agents, and more recently, as treatment for rheumatoid arthritis and psoriasis (Matherly *et al*, 1987; Weinblatt, 1995; Kamen, 1997).

The following literature review focuses on folate biochemistry and one-carbon metabolism with emphasis on its cellular compartmentation. Details of studies of higher plant systems are highlighted, where possible. For convenience, the Introduction is divided into three sections. First, the biochemistry of folates including the biosynthesis of tetrahydrofolate is described. Second, one-carbon metabolism is examined to include the generation, interconversion, and utilization of one-carbon units. Last, the compartmentation of one-carbon metabolism in eukaryotic systems is discussed along with its significance to the present study of higher plant cells.

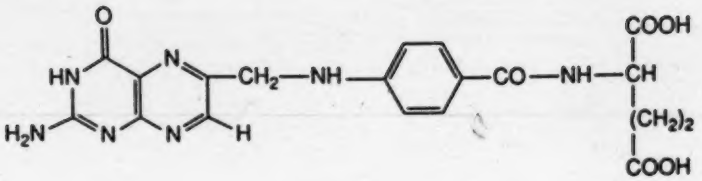
1.1. The Biochemistry of Folates

1.1.1. The chemical nature of naturally occurring folates

The discovery and subsequent characterization of folates involved several independent lines of investigation. First identified as an effective agent in the treatment of tropical macrocytic anemia (Wills, 1931), it was fifteen years before purification of factors responsible for the nutritional effects seen associated with vitamin M (Day *et al*, 1938), vitamin U (Stokstad and Manning, 1938), vitamin B_c (Hogan and Parrott, 1940), norite eluate factor (Snell and Peterson, 1940), folic acid (Mitchell *et al*, 1941), and *Lactobacillus casei* factor (Day *et al*, 1945), revealed that all possessed a *N*-[4-{[(2-amino-4-hydroxy-6-pteridinyl)methyl]amino}benzoyl] glutamic acid structure (Figure 2a). The chemists who elucidated the structure proposed for it the name "pteroylglutamic acid" (Angier *et al*, 1946), though the earlier term "folic acid" (Mitchell *et al*, 1941) is still commonly used. The molecule is comprised of three moieties. An aromatic pteridine ring is linked by a C^9—N^{10} bond to *p*-aminobenzoic acid (*p*-ABA), which in turn is coupled, via an amide bond through its carboxyl moiety, to the amino group of L-glutamic acid.

Folate is a general term used to describe a class of closely related compounds that are similar in structure to folic acid, the synthetic pharmaceutical form of this vitamin. With few exceptions, the role of folate is to carry C_1 units. However, this can only occur when the coenzyme is in the fully reduced state. Therefore, it is not surprising that studies of living organisms have demonstrated that folates tend to exist

A.



B.

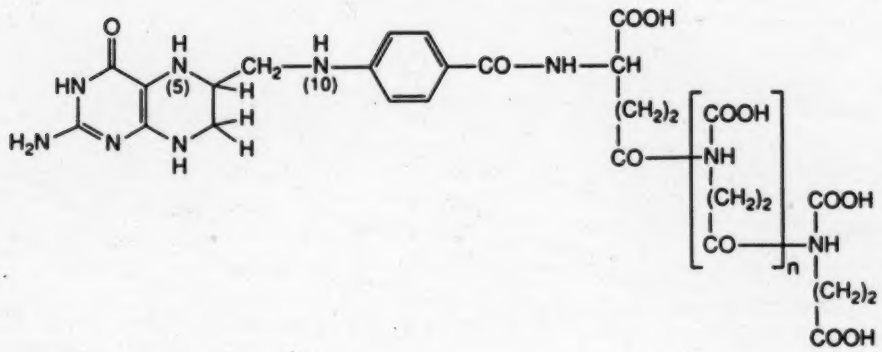


Figure 2. *Structure of: A. Pteroylglutamate and B. Tetrahydropteroyl-polyglutamate.*

Cellular folates can be found substituted at the N^5 position to give the 5-methyl and 5-formyl derivatives, at the N^{10} position to give 10-formyl derivatives, or as bridged compounds to give the N^5,N^{10}-methylene ($-CH_2-$) or methenyl ($=CH^+-$) derivatives. Naturally occurring folates exist predominantly as polyglutamated compounds, containing up to eight γ-glutamyl residues.

in reduced forms (Blakley, 1969). In this regard, the tetrahydrofolate molecule (H_4PteGlu), reduced at positions N^5, C^6, C^7, and N^8 of the pteridine ring (Figure 2b), usually represents the bulk of cellular folates. In metabolically important folates, C_1 substitution occurs by the oxidation and reduction of carbon units at the N^5 and N^{10} positions to produce 5-methyl, 5-formyl, 10-formyl, 5,10-methylene, and 5,10-methenyl derivatives (Blakley, 1969; Cossins, 1984; MacKenzie, 1984). It is important not to confuse the oxidation level of the folate derivative with that of the C_1 unit.

Accumulated evidence suggests that most cellular folates exist as glutamyl conjugated derivatives or tetrahydropteroylpolyglutamates (Figure 2b; McGuire and Coward, 1984; Shane, 1989). Polyglutamyl derivatives, with two to eight glutamate residues, appear ubiquitous in nature, having been described in bacteria, yeast, higher plants, and mammals (McGuire and Bertino, 1981; McGuire and Coward, 1984; Zheng *et al*, 1992). The second and subsequent glutamate moieties are linked to one another by amide bonds through the γ-carboxyl group (Pfiffner *et al*, 1946; Semb *et al*, 1949). It is now clear that the polyglutamate chain aids in the cellular and organellar retention of folates as well as the channeling of substrates between multi-functional protein complexes that catalyze folate-dependent syntheses (Schirch and Strong, 1989; Shane, 1989; Appling, 1991). Folylpolyglutamates are also the preferred substrates for most folate-dependent enzymes. As a result, these enzymes exhibit greater affinities for the polyglutamate molecule as compared to their

monoglutamate counterpart (McGuire and Coward, 1984; Schirch and Strong, 1989; Shane, 1989). Despite possessing the chemical diversity to exist in over 100 distinct and natural forms, less than 50 principal folates exist in most plant and animal tissues (Gregory, 1989).

The growth-promoting effect of folate derivatives on bacteria formed the basis of early microbiological assays that centered on the purification of the vitamin from natural sources. Refinement of these early assays was achieved by exploiting the specificity of the bacterial growth response (Table 1). Modern assays employ *Lactobacillus rhamnosus* (ATCC 7469, formerly *L. casei* subspecies *rhamnosus*) to detect all naturally occurring folates. *Pediococcus acidilactici* (ATCC 8081, formerly *P. cerevisiae*) is used to assay highly reduced folate derivatives that are not methylated, while *Enterococcus hirae* (ATCC 8043, formerly *Streptococcus faecium*) is used to estimate PteGlu derivatives that are not methylated. These organisms do not respond quantitatively to folylpolyglutamates (Tamura, 1990). Therefore, samples containing polyglutamate derivatives are usually hydrolyzed with γ-glutamyl hydrolase (GGH, EC 3.4.19.9) prior to assay (Krumdieck and Baugh, 1969). Hydrolases from a variety of tissues including chicken pancreas (Leichter *et al*, 1977), hog kidney (Olson *et al*, 1948), human or rat plasma (Wolff *et al*, 1949; Horne *et al*, 1981b), and pea cotyledons (Lin *et al*, 1993b), have been used in different laboratories. If the *L. rhamnosus* response increases after enzyme treatment, the folate sample contains derivatives with greater than three glutamate moieties (Tamura

Table 1. *Responses of L. rhamnosus, P. acidilactici, and E. hirae to Various Pteroylglutamates.*

Derivative	Growth activity		
	L. rhamnosus	*P. acidilactici*	*E. hirae*
PteGlu	+	-	+
(6*S*)-H_4PteGlu	+	+	+
(6*S*)-5-CH_3-H_4PteGlu	+	-	-
(6*S*)-5-HCO-H_4PteGlu	+	+	+
(6*S*)-10-HCO-H_4PteGlu	+	+	+

Minus symbol indicates a response < 5% of the maximum. Data from Sauberlich and Baumann (1948), Bakerman (1961), Silverman *et al* (1961), and Bird *et al* (1965).

et al, 1972). On the other hand, GGH treatment of derivatives that have more than two glutamate residues increases the growth response of *P. acidilactici* and *E. hirae* (Tamura, 1990). Complicating the study of folates from biological materials is the extreme lability of folates. The C^9—N^{10} bond is readily cleaved under a variety of conditions with exposure to light, oxygen, elevated temperatures, and extremes of pH having the greatest effects (Blakley, 1969; O'Broin *et al*, 1975; Cooper *et al*, 1978; Kesavan *et al*, 1986). As a result, a number of different methods have been developed to prevent the oxidation and interconversion of the folates during tissue extraction (Tamura, 1990; Mullin and Duch, 1992).

Modern analyses of folates may also employ other methods that are generally less sensitive. These methods include colorimetry, fluorometry, radioassay, radioenzymatic assay, and radioimmunoassay. In addition, some folate assays include bioautography, electrophoresis, gel filtration, and ion exchange-, thin layer-, and high performance liquid chromatographic methods to facilitate the isolation of naturally occurring derivatives (Blakley, 1969; Cossins, 1984; Cossins and Chen, 1997).

1.1.2. The biosynthesis of tetrahydrofolate

The generation of pteridine precursors from guanosine-5'-triphosphate (GTP), the formation and reduction of dihydrofolate (H_2PteGlu), and glutamyl conjugation of tetrahydrofolate for H_4PteGlu$_n$ synthesis, all represent stages of a complex pathway found in plants and most microorganisms (Figure 3). The biosynthesis of 2-amino-4-

Guanosine-5′-triphosphate (GTP)

↓ (1)

L-*threo*-neopterin 2′,3′-cyclic phosphate

↓

6-hydroxymethyl dihydroneopterin

↓ (2)

2-amino-4-hydroxy-6-hydroxymethyl dihydropteridine

ATP ↓ (3)

2-amino-4-hydroxy-6-pyrophosphorylmethyl dihydropteridine

p-ABA ↓ (4)

Dihydropteroate (H_2Pte)

glutamate, ATP ↓ (5)

Dihydrofolate ($H_2PteGlu$)

NADPH ↓ (6)

Tetrahydrofolate ($H_4PteGlu$)

glutamate, ATP ↓ (7)

Tetrahydrofolate polyglutamate ($H_4PteGlu_n$)

Figure 3. *Biosynthesis of Tetrahydropteroylpolyglutamate from Pterin Precursors.* The enzymes involved are: 1. GTP cyclohydrolase I (EC 3.5.4.16), 2. dihydroneopterin aldolase (EC 4.1.2.25), 3. hydroxymethyl dihydropterin pyrophosphokinase (HPPK, EC 2.7.6.3), 4. dihydropteroate synthase (DHPS, EC 2.5.1.15), 5. dihydrofolate synthetase (DHFS, EC 6.3.2.12), 6. dihydrofolate reductase (DHFR, EC 1.5.1.3), and 7. folylpolyglutamate synthetase (FPGS, EC 6.3.2.17).

hydroxy-6-hydroxymethyl dihydropteridine involves the conversion of GTP through hydroxymethyl dihydroneopterin (Brown *et al*, 1975). This is followed by its conversion through a pyrophosphate intermediate to dihydropteroate (H_2Pte), which is itself converted to $H_2PteGlu$ followed by $H_4PteGlu$ and $H_4PteGlu_n$. The sequential operation of the five major biosynthetic enzymes (Figure 3, reactions 3-7) has been reviewed by Cossins and Chen (1997).

Recent enzyme and molecular studies indicate that the biosynthetic pathway of plants, like that of *Escherichia coli*, involves the intermediary formation of H_2Pte (Cossins and Chen, 1997). This synthesis was found to occur exclusively in the mitochondria of pea leaves (Rébeillé *et al*, 1997) with a bifunctional protein possessing HPPK (Figure 3, reaction 3) and DHPS (Figure 3, reaction 4) activities. Consistent with the intracellular localization of this complex, molecular cloning resulted in the isolation of a cDNA with a nucleotide sequence coding for a putative mitochondrial transit peptide (Rébeillé *et al*, 1997). Furthermore, a homodimeric, bifunctional DHFR (Figure 3, reaction 6)-TS (Figure 1, reaction 9) complex was purified to homogeneity from pea leaf mitochondria (Neuburger *et al*, 1996). Observing very low or no activity of the biosynthetic enzymes in compartments other than the mitochondria, these workers suggested that mitochondria are the major, if not unique, site for folate biosynthesis in higher plant cells (Neuburger *et al*, 1996). In agreement with this conclusion, previous studies have indicated a major portion of

HPPK, DHPS, and DHFS (Figure 3, reaction 5) activities is localized in pea seedling mitochondria (Okinaka and Iwai, 1970; Ikeda and Iwai, 1975).

The second principal step, catalyzed by DHPS, is the target of antimicrobial agents. As structural analogues of *p*-ABA, sulfonamides, such as sulfanilamide, competitively inhibit the synthesis of $H_4PteGlu_n$ in microorganisms (Shiota, 1984; Hong *et al*, 1995). Because mammals lack the enzymes prior to DHFR that are required to synthesize pteroylpolyglutamates, they are unaffected by sulfonamides. This accounts for the usefulness of these alternative substrates. As a result, considerable efforts have been made to characterize these bacterial proteins (Bognar *et al*, 1987; Dallas *et al*, 1992).

The product of DHPS, dihydropteroate, is modified by the addition of a glutamate moiety to form dihydrofolate in a reaction catalyzed by DHFS. The distribution of DHFS has been examined in cell-free extracts of *E. coli*, *Bacillus megaterium*, *Corynebacterium* sp., *Neurospora crassa*, and *Saccharomyces cerevisiae*, in addition to other microorganisms (Griffin and Brown, 1964). The enzyme of *N. crassa* has recently been characterized by McDonald *et al* (1995). Also, the existence of DHFS activity has been shown in the leaves of pea, lettuce, cabbage, spinach, and swiss chard (Ikeda and Iwai, 1970). These studies indicate that these organisms were capable of folate synthesis *de novo*. On the other hand, certain bacteria including *L. rhamnosus*, *P. acidilactici*, and *E. hirae*, birds, and mammals, require folate supplementation owing to a deficiency in DHFS (Iwai *et al*, 1977).

The reduction of PteGlu or H_2PteGlu to H_4PteGlu by DHFR has probably received the most attention of all the reactions involved in folate metabolism (for review, see Blakley, 1984). In large part, this is due to its being a target in cancer chemotherapy. While it is incorrect to suggest that DHFR is ubiquitous, this enzyme is widely distributed in nature and present in all dividing cells. The intracellular distribution of DHFR activity has been demonstrated in both the cytosol and mitochondria of yeast (Zelikson and Luzzati, 1977) and rat liver (Brown *et al*, 1965), though the majority of the activity in rat liver was cytoplasmic and microsomal; only approximately 12% of the total enzyme was attributed to a deoxycholate-treated mitochondrial fraction (Brown *et al*, 1965). A later study however, failed to confirm DHFR activity in rat liver mitochondria that were disrupted by sonication (Wang *et al*, 1967). While the nuclei were found to contain negligible amounts of DHFR, they are speculated to contain appreciable levels of the enzyme for regeneration of H_4PteGlu used for thymidylate synthesis in the nuclei (Brown *et al*, 1965).

In higher plants, DHFR exists not as a monomeric protein as in bacteria, fungi, and mammals, but rather as a complex with TS activity. The *dhfr-ts* genes that encode DHFR-TS complexes in *Arabidopsis thaliana* (Lazar *et al*, 1993), *Daucus carota* (Luo *et al*, 1993), and *Glycine max* (Wang *et al*, 1995), have been cloned and sequenced. The deduced amino acid sequences of the DHFR and TS domains, located at the amino and carboxy termini respectively, show striking similarities to those reported for the corresponding monofunctional enzymes of other eukaryotes.

Studies with *A. thaliana* (Lazar *et al*, 1993) and *D. carota* (Luo *et al*, 1997) have also revealed evidence for two distinct genes, each capable of encoding a DHFR-TS complex. As plant cells require DNA precursors in nuclei, mitochondria, and chloroplasts, the possibility that different DHFR-TS complexes may occur in these compartments arises. Though this has yet to be fully investigated, a plastidial DHFR-TS complex in carrot was demonstrated using immunogold labeling (Luo *et al*, 1997).

With a few exceptions seen in bacteria (Blakley, 1984; Fleming and Schilsky, 1992), DHFR proteins are strongly inhibited by antifolates such as aminopterin and methotrexate (MTX). These dihydrofolate analogues block DHFR resulting in $H_4PteGlu_n$ deficiencies. In mammalians, MTX cytotoxicity is enhanced by glutamyl conjugation (Fleming and Schilsky, 1992), a reaction catalyzed by FPGS (Figure 3, reaction 7). In contrast, much is still to be elucidated about the action of MTX on plant cells (Imeson *et al*, 1988; Crosti *et al*, 1993; Wu *et al*, 1994).

Observations that all organisms are capable of synthesizing folylpolyglutamates, regardless of whether or not they are able to synthesize folate *de novo*, are indicative of the vital role that $H_4PteGlu_n$ plays in cellular metabolism. Generally, folylpolyglutamates are the major intracellular forms of folate. As the chain length of these intracellular polyglutamates always exceeds the longest form that can be transported efficiently across cellular membranes, all cells contain FPGS (McGuire and Coward, 1984; Shane, 1989). The importance of these derivatives in one-carbon metabolism is also supported by work performed on mutant cell lines.

Lesions which affect the expression of FPGS result in auxotrophies for products of one-carbon metabolism (Shane, 1989). Shane's group has recently focused on a FPGS-deficient mammalian cell line (AUX B1; Sussman *et al*, 1986; Shane *et al*, 1990; Garrow *et al*, 1992; Lowe *et al*, 1993; Lin *et al*, 1993a; Kim *et al*, 1993). Transformations on this line with either the human or *E. coli* FPGS gene demonstrate that eukaryotic cells normally express cytosolic and mitochondrial FPGS proteins encoded by the same nuclear gene. Working with wild-type Chinese hamster ovary (CHO) cells, Lin *et al* (1993a) observed approximately 50% of the enzyme activity in the mitochondria. Other investigators have demonstrated most of the synthetase activity is localized in the cytoplasm of sheep and rat liver cells (~ 75%), with lower activities detected in the nuclear, mitochondrial, lysosomal, and microsomal fractions (Gawthorne and Smith, 1973; McGuire *et al*, 1979). This parallels the subcellular distribution of folate derivatives as determined by microbiological assay (Gawthorne and Smith, 1973; Shin *et al*, 1976). Similarly, plant cells have been shown to express cytosolic (Chan *et al*, 1986) and mitochondrial (Neuburger *et al*, 1996) FPGS activities. However, the relative contribution of each form to folate biosynthesis and one-carbon metabolism has yet to be determined. Also, the FPGS gene from higher plants has still not been cloned or sequenced.

The FPGS found in some bacteria including *E. coli* and *Corynebacterium* sp., exists as a bifunctional protein with DHFS activity encoded by the *folC* gene (Shane, 1980; Ferone and Warskow, 1983; Bognar *et al*, 1985). In contrast, fungi, plants,

and mammals have monofunctional, monomeric proteins that synthesize polyglutamates (for review, see Cossins and Chen, 1997). Synthesis of the polyglutamate chain is affected by the incubation time and substrate concentration (McGuire and Coward, 1984; Cichowicz and Shane, 1987; Imeson *et al*, 1990). Therefore, incubations carried out for extended periods of time (e.g., 24 h) with non-saturating amounts of H_4PteGlu, tend to result in longer polyglutamate products.

1.2. Pathways of One-carbon Metabolism

1.2.1. The glycine cleavage system

The glycine decarboxylase (GDC) reaction (Figure 1, reaction 10; eqn. 1)

$$\text{Glycine} + H_4\text{PteGlu}_n + \text{NAD} \rightarrow 5,10\text{-}CH_2\text{-}H_4\text{PteGlu}_n + NH_3 + CO_2 + \text{NADH} \quad (1)$$

represents a major source of 5,10-methylene-H_4PteGlu$_n$ in most prokaryotic and eukaryotic species (Cossins, 1980; Schirch, 1984). In higher plants, fungi, and animals, the reaction is catalyzed by a complex of mitochondrial matrix proteins (Schirch, 1984; Douce and Neuburger, 1989; Douce *et al*, 1994). The complex is comprised of four subunits which include a homodimeric, pyridoxal phosphate-binding protein (P-protein, M_r ~ 98 kDa), a monomeric lipoamide-containing peptide (H-protein, M_r ~ 15 kDa), a tetrahydrofolate-binding monomeric protein (T-protein, M_r ~ 45 kDa), and a homodimeric lipoamide dehydrogenase protein (L-protein, M_r ~

59 kDa). The estimated stoichiometry of the complex is 2 P-protein dimers, 27 H-protein monomers, 9 T-protein units, and 1 L-protein dimer (Oliver *et al*, 1990).

The GDC reaction is irreversible *in vivo* and favors glycine oxidation (Wagner, 1996). Therefore, a rather large pool of 5,10-CH_2-H_4PteGlu$_n$ must accumulate in the matrix space (Bourguignon *et al*, 1988). Under *in vitro* conditions, this derivative accounts for approximately 65-80% of the folate pool and drives the reversible serine hydroxymethyltransferase (SHMT) reaction (Figure 1, reaction 7) in the direction of serine synthesis (Rebeille *et al*, 1994). Although both enzymes have greater affinity for polyglutamate substrates as opposed to monoglutamates (Besson *et al*, 1993; Rebeille *et al*, 1994), there is no direct evidence that 5,10-CH_2-H_4PteGlu$_n$ is channeled between the two closely related reactions. However, the shared tetrahydrofolate pool in the matrix indicates that a biochemical link exists between the glycine cleavage system and SHMT (Bourguignon *et al*, 1988). Earlier studies suggested that two molecules of glycine are consumed for every mole of serine produced during photorespiration (Cossins, 1980). However, it has yet to be determined whether all of the 5,10-CH_2-H_4PteGlu$_n$ pool, formed during photorespiration, is used for the synthesis of serine or whether it can be used to generate other folate derivatives such as 5-CH_3-, 5-HCO-, and 10-HCO-H_4PteGlu$_n$.

The oxidation of glycine by mitochondria represents an important step in the metabolic pathway of photorespiration in leaf tissue (Lorimer and Andrews, 1980; Osmond, 1981). The major role this reaction plays in photorespiration has resulted in

the intense study of the structure, function, and biogenesis of GDC in higher plant mitochondria. The individual genes encoding GDC subunit proteins have recently been cloned in several laboratories. For example, the P-protein was isolated, cloned, and characterized using a pea leaf cDNA library (Turner *et* al, 1992a). The cDNA encoding the H-protein in peas has been cloned and sequenced (Kim and Oliver, 1990). Cloning and characterization of the T-protein have been accomplished by screening a λgt11 pea leaf cDNA library (Bourguignon *et al*, 1993). The cDNA for the complete L-protein precursor was isolated and characterized and these investigators also showed that the L-protein was a functional component of several other mitochondrial enzyme complexes (Bourguignon *et al*, 1992). The expression of genes encoding the four subunits during pea leaf development is negligible during the initial stages but increases dramatically once the primary leaflet opens (Vauclare *et al*, 1996). Transcription of GDC genes was coordinated and occurred early, with a peak at 7 d, a stage at which mitochondria are still unable to oxidize glycine (Vauclare *et al*, 1996).

In related work, Walker and Oliver (1986) have shown that the mitochondria of light-grown pea leaves have 10-fold greater levels of GDC than mitochondria from etiolated leaves and this difference is substantiated by the amounts of the four subunit proteins. In fact, after centrifugation on a Percoll gradient, mitochondria from etiolated leaves have been observed to be located higher in the tube than mitochondria from mature leaves containing the GDC complex. This suggests that mitochondria

from etiolated leaves are lighter as GDC represents a large (~ 30-50%) proportion of the matrix protein in green leaf mitochondria (Day *et al*, 1985; Bourguignon *et al*, 1988). In contrast, the GDC of mammalian mitochondria represents a minor fraction of the total matrix protein (Hampson *et al*, 1983). Light-grown pea seedlings had significantly more mRNA for the H-protein than dark-grown control plants and an increase in transcription occurred in the latter plants upon transfer to white light (Kim and Oliver, 1990). Similarly, the induction of mRNAs encoding the P (Turner *et al*, 1992a) and L (Turner *et al*, 1992b) proteins of GDC have been demonstrated to be affected by light. The levels of mRNA for the T-protein are also lower (8-10 fold) in etiolated leaves than those that are green (Bourguignon *et al*, 1993). Interestingly, this same group failed to detect any trace of H-protein in the roots while the other three subunit proteins were detectable by Northern and Western blot analyses.

1.2.2. Folate-dependent serine metabolism

The serine hydroxymethyltransferase reaction (eqn. 2) results in the

$$\text{Serine} + H_4\text{PteGlu}_n \leftrightarrow \text{glycine} + 5,10\text{-}CH_2\text{-}H_4\text{PteGlu}_n \quad (2)$$

interconversion of serine and glycine. The majority of cellular 5,10-CH_2-H_4PteGlu$_n$, generated by SHMT, is utilized for the synthesis of thymidylate, methionine, purines, and choline (for review, see Schirch, 1984). The enzyme has been purified and

characterized from several prokaryotic and eukaryotic sources including plants (Schirch, 1984; Cossins, 1987).

Unlike the GDC complex which is strictly a mitochondrial enzyme, SHMT activity is also present in the cytoplasm of eukaryotes (Schirch, 1984; Appling, 1991), and chloroplasts and nuclei of plants (Cossins, 1980; Besson *et al*, 1995; Neuburger *et al*, 1996). However root tissue from pea, despite possessing high SHMT levels, lacks the mitochondrial forms of this protein (Turner *et al*, 1992c). Using spinach leaf protoplasts, Besson *et al* (1995) found SHMT activity to be distributed between three cellular compartments. Mitochondrial activity constituted approximately 50% of the total cellular activity while the chloroplastic and cytosolic compartments contained about 20-25% each. Studies of plant SHMT have focused on the mitochondrial isoenzyme of photorespiring leaves (Bourguignon *et al*, 1988; Vijaya *et al*, 1991; Turner *et al*, 1992c and 1993; Besson *et al*, 1993 and 1995; Rebeille *et al*, 1994; Kopriva and Bauwe, 1995). This protein has been isolated and purified to apparent homogeneity from pea leaves and exists as a tetramer of four identical subunits of 53 kDa (Bourguignon *et al*, 1988). Immunological studies have revealed two distinct forms of SHMT from pea leaf mitochondria and a third, non-mitochondrial form (Turner *et al*, 1992c). The deduced amino acid sequence of the pea mitochondrial SHMT has homologies to the cytosolic and mitochondrial SHMT isoforms of rabbit (Martini *et al*, 1987 and 1989) as well as to the mitochondrial SHMT of *Solanum tuberosum* (Kopriva and Bauwe, 1995).

The pea leaf mitochondrial SHMT has been shown to be associated in the matrix, with the GDC complex (Bourguignon *et al*, 1988). The continuous operation of the multienzyme complex is ensured by NADH reoxidation through the mitochondrial electron transport chain or by matrix malate dehydrogenase working in the reverse direction (Douce and Neuburger, 1989) and by recycling of 5,10-CH_2-$H_4PteGlu_n$ to $H_4PteGlu_n$. Therefore, the SHMT reaction must be continuously driven towards the production of serine, the unfavorable direction {K_{eq} = [glycine][5,10-CH_2-$H_4PteGlu_n$] / [serine][$H_4PteGlu_n$] = 12-19 (Besson *et al*, 1993)}. In fact, studies on a *A. thaliana* mutant deficient in mitochondrial SHMT activity suggest that this pathway is the sole source of photorespiratory CO_2 release and that this activity is not essential for any other function within the plant (Somerville and Ogren, 1981).

In common with GDC, the mRNA encoding the mitochondrial SHMT in pea leaves is strongly induced when etiolated plants are exposed to light (Turner *et al*, 1993). As well, the level of this mRNA species declines dramatically when mature plants are placed in the dark for 48 hours. While these findings strongly suggest that light controls the expression of mitochondrial SHMT in leaf tissue, it is not clear whether other regulatory mechanisms exist. Plant tissues contain 5-HCO-$H_4PteGlu_n$ (Cossins, 1980) which is a strong inhibitor of bacterial, fungal, and mammalian SHMT proteins (Stover and Schirch, 1991; Kruschwitz *et al*, 1994). Therefore, it is possible that a mitochondrial pool of 5-HCO-$H_4PteGlu_n$ in plants could exert some control over the SHMT reaction. There is little information on the one-carbon

substitution status of mitochondrial folates in plants and the sensitivity of the SHMT reaction to inhibition by 5-HCO-H_4PteGlu$_n$. As a result, it is still not possible to judge if a regulatory mechanism based on this formyl folate has importance in plant mitochondria. Given the fact that glycine decarboxylase and SHMT are major folate-dependent proteins in pea leaf mitochondria (Bourguignon *et al*, 1988), the greening process may induce profound changes in the folate pool. Prior to the present studies, this had not been elucidated.

1.2.3. The interconversion of one-carbon units

1.2.3.1. The interconversion of 5-HCO- and 5,10-CH^+-H_4PteGlu$_n$

Whole organisms and cultured cells commonly utilize 5-HCO-H_4PteGlu$_n$ as a source of folate (MacKenzie, 1984). This derivative, formed irreversibly from 5,10-CH^+-H_4PteGlu$_n$ by SHMT in the presence of glycine (Figure 1, reaction 7; Stover and Schirch, 1990), is a potent inhibitor of several folate-dependent enzymes. These include SHMT, DHFR, 5,10-CH_2-H_4PteGlu$_n$ dehydrogenase (DHY; Figure 1, reaction 8), 5,10-CH^+-H_4PteGlu$_n$ cyclohydrolase (CYC; Figure 1, reaction 5), aminoimidazole carboxamide ribonucleotide (AICAR) transformylase (Figure 1, reaction 3), and methionyl-tRNA transformylase (Figure 1, reaction 4) (Stover and Schirch, 1992). Clearly, 5-HCO-H_4PteGlu$_n$ has potential to regulate folate-dependent metabolic pathways.

The only enzyme known to metabolize 5-HCO-$H_4PteGlu_n$ is 5,10-CH^+-$H_4PteGlu_n$ synthetase (Figure 1, reaction 6; eqn. 3). This reaction allows 5-HCO-

5-HCO-$H_4PteGlu_n$ + ATP $\rightarrow$ 5,10-CH^+-$H_4PteGlu_n$ + ADP + P_i (3)

$H_4PteGlu_n$ to participate in one-carbon metabolism. The combined activities of 5,10-CH^+-$H_4PteGlu_n$ synthetase and SHMT appear to constitute a futile cycle that may buffer cellular 5-HCO-$H_4PteGlu_n$ concentrations (Girgis *et al*, 1997). The synthetase has been purified to homogeneity from both prokaryotic and eukaryotic sources (Stover and Schirch, 1993) but to date, has not been reported in plant species. The 5,10-CH^+-$H_4PteGlu_n$ synthetase of rabbit liver is cytoplasmic (Maras *et al*, 1994) whereas human liver contains 85% of the synthetase activity in the cytosol and the remainder in the mitochondria (Bertrand *et al*, 1995).

1.2.3.2. The catabolism of 10-HCO-$H_4PteGlu_n$

10-HCO-$H_4PteGlu_n$ dehydrogenase (EC 1.5.1.6) catalyzes the irreversible NADP-dependent oxidation of 10-HCO-$H_4PteGlu_n$ to $H_4PteGlu_n$ and CO_2 (eqn. 4).

10-HCO-$H_4PteGlu_n$ + H_2O + NADP $\rightarrow$ $H_4PteGlu_n$ + CO_2 + NADPH (4)

The purified hepatic proteins of rat (Scrutton and Beis, 1979; Min *et al*, 1988; Cook *et al*, 1991), pig (Rios-Orlandi *et al*, 1986; Min *et al*, 1988), and rabbit (Schirch *et al*, 1994), also exhibit lower levels of a NADP-independent hydrolase activity that converts 10-HCO-H_4PteGlu$_n$ to H_4PteGlu$_n$ and formate (eqn. 5), though it is

$$\text{10-HCO-}H_4\text{PteGlu}_n + H_2O \rightarrow H_4\text{PteGlu}_n + \text{formate} \quad (5)$$

uncertain whether simple hydrolysis of 10-HCO-H_4PteGlu$_n$ occurs in the cell. The physiological importance of these two reactions is not clear. However, it has been postulated that 10-HCO-H_4PteGlu$_n$ dehydrogenase represents an important site for binding folylpolyglutamates and for regulating the interconversion of 10-HCO-H_4PteGlu$_n$ and H_4PteGlu$_n$ (Krebs *et al*, 1976; Champion *et al*, 1994; Bässler, 1997). It has also been suggested that 10-HCO-H_4PteGlu$_n$ dehydrogenase, which exists in an enzyme cluster with SHMT (Wagner, 1996), plays a role in removing formate during methanol toxicity (Neymeyer and Tephly, 1994). To date, the existence of these enzyme activities in plants has not been assessed.

1.2.3.3. C_1-THF synthase activities of higher plants

As glycine and serine represent major sources of one-carbon units in most cells, entry of C_1 units into one-carbon metabolism is heavily dependent on the operation of GDC and SHMT. The cleavage of either amino acid produces 5,10-

CH_2-H_4PteGlu$_n$ which can participate directly in the synthesis of thymidylate. Alternatively, 5,10-CH_2-H_4PteGlu$_n$ may be reduced to 5-CH_3-H_4PteGlu$_n$ for methionine biosynthesis (Figure 1, reactions 11 and 12) or oxidized to 10-HCO-H_4PteGlu$_n$ for use in purine synthesis (Figure 1, reactions 2 and 3). This latter derivative, also formed by the activation of formate (Figure 1, reaction 1; Cossins, 1987; Appling, 1991), must be reduced prior to involvement in the synthesis of thymidylate, methionine, and serine (for review, see MacKenzie, 1984). Thus, the interconversion of 5,10-CH_2-H_4PteGlu$_n$ and 10-HCO-H_4PteGlu$_n$ is central to the utilization of one-carbon units and is accomplished by three reversible reactions: 5,10-CH_2-H_4PteGlu$_n$ dehydrogenase (eqn. 6), 5,10-CH^+-H_4PteGlu$_n$ cyclohydrolase (eqn. 7), and 10-HCO-H_4PteGlu$_n$ synthetase (SYN, eqn. 8).

5,10-CH_2-H_4PteGlu$_n$ + NADP $\leftrightarrow$ 5,10-CH^+-H_4PteGlu$_n$ + NADPH (6)

5,10-CH^+-H_4PteGlu$_n$ + H_2O $\leftrightarrow$ 10-HCO-H_4PteGlu$_n$ + H^+ (7)

Formate + H_4PteGlu$_n$ + ATP $\leftrightarrow$ 10-HCO-H_4PteGlu$_n$ + ADP + P_i (8)

In yeast and mammalian cells, DHY, CYC, and SYN activities are associated with a single trifunctional protein (MacKenzie, 1984), designated C_1-tetrahydrofolate synthase (C_1-THF synthase). Cytosolic and mitochondrial forms of this protein, encoded by separate genes, have been characterized (MacKenzie, 1984). In addition, a monofunctional, NAD-dependent DHY protein has been identified in the cytosol of

yeast species (Barlowe and Appling, 1990; West *et al*, 1993 and 1996). The mitochondria of human (Peri *et al*, 1989) and mouse (Bélanger and MacKenzie, 1989) cells also possess a NAD-dependent DHY protein that contains CYC activity.

In contrast, these three activities are associated with monofunctional proteins in a number of bacteria (Whitehead and Rabinowitz, 1988; Lovell *et al*, 1990). On the other hand, *E. coli* (Dev and Harvey, 1978; D'Ari and Rabinowitz, 1991), *Clostridium thermoacetium* (Ljungdahl *et al*, 1980), and *Photobacterium phosphoreum* (Pawelek and MacKenzie, 1996) express a bifunctional DHY-CYC complex that lacks SYN activity. The DHY activity of these proteins is NADP-dependent except for that of *P. phosphoreum* which uses either NADP or NAD as a cofactor. Monofunctional NAD- or NADP-dependent DHY have also been purified from *Acetobacterium woodii* (Tanner *et al*, 1978) and *C. formicoacetium* (Ljungdahl *et al*, 1980).

Early studies show that these three activities are present in significant levels of many plant tissues (for review, see Cossins, 1980). The cotyledons (Kirk *et al*, 1995) and leaves (Chen *et al*, 1997) of pea, and the leaves of spinach (Nour and Rabinowitz, 1991), bean, wheat, corn, and barley (Kirk *et al*, 1995), express a DHY-CYC complex that lacks SYN activity. In pea tissues, these three activities are mainly associated with the cytosolic fraction (Kirk *et al*, 1994 and 1995; Chen *et al*, 1997), with less than 1% of total activity attributed to the mitochondria (Chen *et al*, 1997). The spinach leaf SYN has structural homologies to the SYN domains of yeast and

mammalian C_1-THF synthase, as well as a putative sequence for binding polyglutamyl folates (Nour and Rabinowitz, 1992). The SYN protein of pea cotyledons occurs as a homodimer (M_r ~ 56 kDa) that uses $H_4PteGlu_n$ as the preferred folate substrate (Kirk *et al*, 1994). NADP-dependent DHY and CYC activities from this source are associated with a bifunctional, homodimeric protein (M_r ~ 38.5 kDa) that does not show a strong preference for polyglutamate substrates (Kirk *et al*, 1995). This latter property is also shown by the corresponding activities of yeast C_1-THF synthase (Rabinowitz, 1983).

The structural organization of these three activities in pea leaves is similar to that found in the cotyledon (Chen *et al*, 1997) though the molecular weights of the two proteins differ slightly (DHY-CYC M_r ~ 38 kDa and SYN M_r ~ 66 kDa). Polyclonal antibodies raised against these two cytosolic proteins were used to detect immunologically related proteins of 38 kDa (with DHY-CYC-Ab), and 40 and 44 kDa (with SYN-Ab), in the mitochondria. Immunoaffinity chromatography indicated that the bulk of mitochondrial SYN activity is not associated with mitochondrial DHY or CYC (Chen *et al*, 1997). The deduced primary structure of pea cytosolic DHY-CYC protein shares most of the conserved domains found in the corresponding enzymes of other species including human, rat, mouse, yeast, and *E. coli* (Chen, 1997).

Similar to GDC and SHMT, light plays a role in the regulation of DHY-CYC and SYN expression (Chen *et al*, 1997). Thus during greening of etiolated pea

seedlings, the specific enzyme activities of DHY-CYC in whole-leaf extracts rose over 2-fold and more DHY-CYC-Ab cross-reacting protein was detected. The specific activity of SYN dropped 5-fold over this greening period and less SYN-Ab cross-reacting protein was detected. The decrease noted for SYN-Ab reacting protein was also apparent with the 44 kDa mitochondrial band and was accompanied by a specific activity decrease of more than 2-fold. In contrast, activity and protein levels of DHY and CYC in mitochondrial extracts were not appreciably different before and after light treatment (Chen *et al*, 1997). In barley leaves, greening has been demonstrated to increase the specific activities of DHY and SYN (Gifford and Cossins, 1982). Furthermore, greening in the presence of α-hydroxy-2-pyridinemethane sulfonate, an inhibitor of glycolate oxidation, reduced this enhancement of SYN activity in barley leaves as well as the ability to metabolize formate. Therefore, formate for the SYN reaction in barley leaves may be partly derived from glycolate during photorespiration (Gifford and Cossins, 1982). Interestingly, Chen *et al* (1997) failed to detect significant levels of C_1-THF synthase activities in chloroplasts. These organelles contain a folate pool that is utilized in the transformylation of methionyl-tRNA (Staben and Rabinowitz, 1984; Cossins, 1987). Furthermore, there is evidence that isolated pea chloroplasts convert formate to serine (Shingles *et al*, 1984) and contain SHMT activity (Cossins, 1980). It follows that chloroplasts should contain C_1-THF synthase activities for the generation and interconversion of one-carbon units needed in these reactions. DHY activity was

indeed detected in pea leaf mitochondria, cytosol, nuclei, and chloroplasts, in an earlier study (Neuburger *et al*, 1996). Clearly, more work is required to justify this apparent discrepancy.

1.2.4. Purine biosynthesis

Folate derivatives have importance in nucleic acid biosynthesis through a direct involvement in the *de novo* synthesis of purines (Rowe, 1984; Henikoff, 1987; Zalkin and Dixon, 1992). The C^2 and C^8 of the purine ring are derived from 10-HCO-H_4PteGlu$_n$ in reactions catalyzed by glycinamide ribonucleotide (GAR) transformylase (eqn. 9) and AICAR transformylase (eqn. 10). In *E. coli* and *B. subtilis*, two distinct GAR transformylases, 10-HCO-H_4PteGlu$_n$- and formate-dependent, are expressed during *de novo* purine biosynthesis (Ebbole and Zalkin, 1987; Smith and Daum, 1987; Nygaard and Smith, 1993; Saxild *et al*, 1994 and 1995). Formate required for the latter reaction appears to be generated by a 10-HCO-H_4PteGlu$_n$ hydrolase (Nagy *et al*, 1993). Eukaryotes commonly possess 10-HCO-H_4PteGlu$_n$-dependent GAR transformylases that are associated with other enzymes of *de novo* purine biosynthesis such as GAR synthetase (EC 6.3.4.13) and

GAR + 10-HCO-H_4PteGlu$_n$ $\rightarrow$ N-formyl-GAR + H_4PteGlu$_n$ (9)

AICAR + 10-HCO-H_4PteGlu$_n$ $\rightarrow$ N-formyl-AICAR + H_4PteGlu$_n$ (10)

aminoimidazole ribonucleotide (AIR) synthetase (EC 6.3.3.1) (Henikoff, 1986; Aimi *et al*, 1990; Zalkin and Dixon, 1992). On the contrary, cDNA encoding a monofunctional GAR transformylase has been isolated from *A. thaliana* (Schnoor *et al*, 1994). In other studies of purine biosynthesis in plants, the mitochondria and plastids of *Vigna unguiculata* root nodules were shown to contain a full complement of enzymes (Atkins *et al*, 1997).

In avian liver, AICAR transformylase is associated with other enzymes of purine biosynthesis (Mueller and Benkovic, 1981) or one-carbon metabolism (Smith *et al*, 1981). A detailed characterization of this protein has yet to be reported for plants but its activity has been detected in the mitochondria and plastids of cowpea nodules (Atkins *et al*, 1997) and in other legumes (for review, see Cossins, 1987).

1.2.5. Thymidylate biosynthesis

One-carbon moieties at the oxidation level of formaldehyde are utilized in the *de novo* biosynthesis of thymidylate. Thymidylate synthase catalyzes the conversion of deoxyuridine monophosphate (dUMP) and 5,10-CH_2-H_4PteGlu$_n$ to deoxythymidine monophosphate (dTMP) and dihydrofolate (eqn. 11). In this reaction,

$$\text{dUMP} + \text{5,10-CH}_2\text{-H}_4\text{PteGlu}_n \rightarrow \text{dTMP} + \text{H}_2\text{PteGlu}_n \qquad (11)$$

5,10-CH_2-H_4PteGlu$_n$ serves as both the one-carbon donor and reducing agent, with the H^6 of the folate derivative being quantitatively transferred to the methyl group of thymidylate (Santi and Danenberg, 1984). This enzyme is unique in that it represents the sole path for *de novo* thymidylate synthesis and is the only folate-dependent enzyme that oxidizes H_4PteGlu$_n$ during a C_1 transfer (Santi and Danenberg, 1984). For the sustained production of dTMP, it is therefore necessary to regenerate the reduced form of folate. This is accomplished by DHFR, a key enzyme in H_4PteGlu$_n$ biosynthesis.

As noted earlier (see section 1.1.2), TS and DHFR occur as separate monofunctional enzymes in bacteria, fungi, and mammals. Some higher plants, protozoans, and green algae however, express a bifunctional DHFR-TS protein. It has been suggested that the TS activity of pea leaves occurs exclusively in the mitochondria (Neuburger *et al*, 1996). There is also good evidence that this complex allows channeling of H_2PteGlu$_n$ from TS to DHFR in protozoa (Ivanetich and Santi, 1990).

1.2.6. Methionine biosynthesis

A major cycle of one-carbon utilization involves the reduction of 5,10-CH_2-H_4PteGlu$_n$ to 5-CH_3-H_4PteGlu$_n$, followed by the transfer of its methyl group to homocysteine to form methionine. The reduction of 5,10-CH_2-H_4PteGlu$_n$ is catalyzed

by 5,10-CH_2-H_4PteGlu$_n$ reductase (eqn. 12), which has been identified in a variety of

5,10-CH_2-H_4PteGlu$_n$ + $FADH_2$ → 5-CH_3-H_4PteGlu$_n$ + FAD (12)

bacterial, fungal, plant, and mammalian sources (for review, see Matthews, 1984). This reaction represents the committed step in methionine synthesis and is regulated by negative feedback inhibition by *S*-adenosylmethionine, which rises in parallel with methionine (Kutzbach and Stokstad, 1971). Recent clinical studies report that deficiencies in 5,10-CH_2-H_4PteGlu$_n$ reductase are a major genetic abnormality in homocysteine metabolism (Rozen, 1996).

The terminal step in the *de novo* biosynthesis of methionine is the transmethylation of homocysteine. In plants, this reaction is catalyzed by a vitamin B_{12}-independent methionine synthase (eqn. 13; for review, see Cossins, 1987). In

5-CH_3-H_4PteGlu$_n$ + L-homocysteine → H_4PteGlu$_n$ + L-methionine (13)

contrast, the corresponding enzymes of mammalian cells and some bacteria are cobalamin-dependent (Matthews, 1984). *E. coli* expresses both cobalamin-independent and cobalamin-dependent synthases (Banerjee *et al*, 1990; Banerjee and Matthews, 1990).

1.2.7. Initiation of protein synthesis

Prokaryotes require 10-HCO-$H_4PteGlu_n$ as the formyl donor in the synthesis of formylmethionyl-tRNA to initiate polypeptide synthesis (Staben and Rabinowitz, 1984). In eukaryotes, the initiation of protein synthesis in mitochondria and chloroplasts also requires fMet-tRNA (Staben and Rabinowitz, 1984). The folate-dependent generation of formylmethionyl-tRNA is catalyzed by methionyl-tRNA transformylase (eqn. 14). The enzyme has been isolated and characterized from a

$$10\text{-HCO-}H_4PteGlu_n + \text{methionyl-tRNA} \rightarrow H_4PteGlu_n + \text{fMet-tRNA} \qquad (14)$$

variety of organisms, including plant species (for review, see Cossins, 1987).

1.3. Compartmentation of Folate Metabolism

Folate-mediated one-carbon metabolism is known to be highly compartmentalized in eukaryotic cells (Figure 4; Appling, 1991; Wagner, 1996). To achieve this compartmentation, folate pathways are sequestered in subcellular organelles (Figure 4) and is described below. The distribution of folate between free and protein-bound states, together with substrate channeling, also serves to segregate intermediates of the pathway into functionally distinct pools. These latter two schemes allow the products of an enzyme-catalyzed reaction to be transferred directly to the next enzyme to act as substrates without diffusing in and out of solvent. This

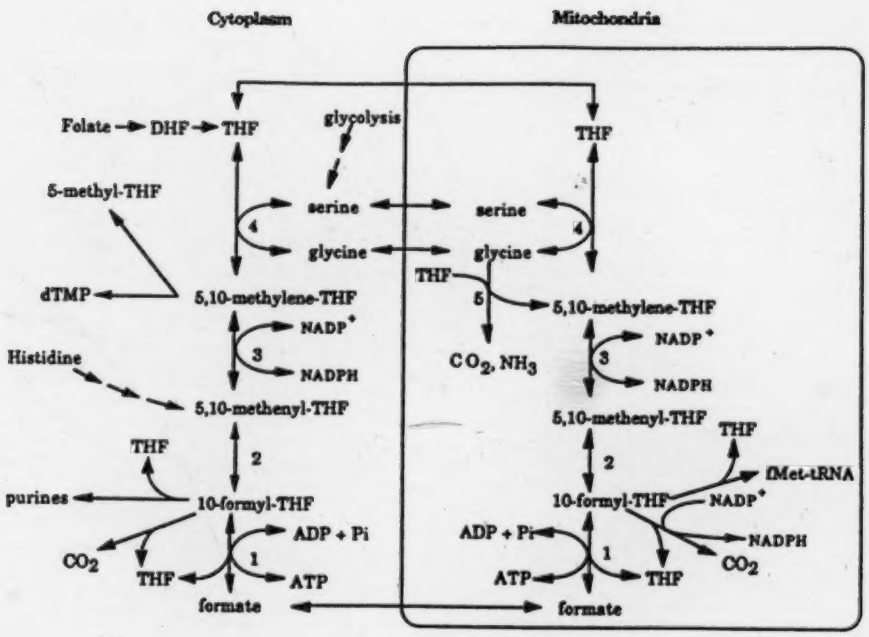


Figure 4. *Compartmentation of Folate-mediated One-carbon Metabolism.*

The scheme is derived with some modifications from Appling (1991) for mammalian and yeast cell systems. The numbered reactions are: 1. SYN, 2. CYC, 3. DHY, 4. SHMT, and 5. GDC. The chloroplasts of plant cells are also involved in folate metabolism (see section 1.2.3.3).

offers a significant advantage to the reduced folate molecules of these reactions as enzyme-bound coenzymes show increased stability. Support for these models has come from the purification of enzymes and development of accurate methods to determine the size of folate pools. Thus in mammalian cells, the concentration of folate-dependent enzymes exceeds that of their folate substrates (Wagner, 1996). This taken together with the fact that pteroylpolyglutamates have dissociation constants in the 100 nM range, suggests that most cellular folates are protein-bound (Schirch and Strong, 1989). This raises the possibility that the rate of folate metabolism may be controlled by the rate of diffusion of substrates and products from these enzymes.

It is also clear that the intracellular compartmentation of folate metabolism occurs mainly between the cytosol and mitochondria. Studies of mouse liver (Swendseid *et al*, 1950) and brain (McClain *et al*, 1975; Carl *et al*, 1980), guinea pig liver (Corrocher and Hoffbrand, 1972) and intestine (Hoffbrand and Peters, 1969), Chinese hamster ovary (Lin *et al*, 1993a), sheep liver (Gawthorne and Smith, 1973), and rat liver (Shin *et al*, 1976; Zamierowski and Wagner, 1977; Cook and Blair, 1979; Horne *et al*, 1989; Balaghi *et al*, 1993) and pancreas (Horne and Holloway, 1997), have shown that intracellular folate is localized primarily in the cytosol (50-80%) and mitochondria (20-50%). Figure 4 depicts how the enzymes of one-carbon metabolism are probably sequestered. It should be noted that some of these enzymes are found in either the cytoplasm or the mitochondria, while others are located in both

compartments. The observed distribution of folate derivatives and folate-requiring enzymes between these two compartments is reflective of the roles performed in each compartment. In the cytosol, folylpolyglutamates assist in the biosynthesis of purines and thymidylate, and in methyl group biogenesis (Appling, 1991). In contrast, folates are required in the mitochondria for the formylation of methionyl-tRNA for organellar protein synthesis (Staben and Rabinowitz, 1984) and the catabolism of glycine (Kikuchi, 1973). Occurring in both compartments is the interconversion of glycine and serine (Schirch, 1984) and of substituted tetrahydrofolates (MacKenzie, 1984). Hence, the majority of cytosolic folates in rat liver are 5-CH_3- and unsubstituted $H_4PteGlu_n$ (Horne *et al*, 1989; Balaghi *et al*, 1993). Furthermore, these two studies report that the latter coenzyme and 10-HCO-$H_4PteGlu_n$ comprise of over 80% of mitochondrial folates, with smaller amounts of 5-HCO- and 5-CH_3-$H_4PteGlu_n$ also present. These results, along with similar profiles recorded in CHO cells (Shane, 1995) and rat pancreas (Horne and Holloway, 1997), clearly demonstrate the distinct pools of tetrahydropteroylglutamate which exist in the cytosol and mitochondria. In plant cells, folates have also been reported in the chloroplasts with the principal derivatives being 5-CH_3-, 10-HCO-, and 5-HCO-$H_4PteGlu_n$ (Shah and Cossins, 1970; Cossins and Shah, 1972). On the other hand, nuclei contain only very low levels of folate (Appling, 1991) and have yet to be studied in detail.

Communication between the cytosolic and mitochondrial folate pools is present but limited. The mitochondrial pool appears to be obtained from the uptake

of folic acid from the cytosol (Zamierowski and Wagner, 1977). A separate study performed on isolated rat liver mitochondria reported that tetrahydrofolates are not transported into the matrix whereas a carrier-mediated mechanism can rapidly transport folic acid, dihydrofolate, and methotrexate (Cybulski and Fisher, 1981). Recently, Horne *et al* (1992) presented evidence that transport of 5-HCO-H_4PteGlu in rat liver mitochondria is a carrier-mediated process that is specific for the reduced forms of folate monoglutamates. Upon entering the mitochondria, this folate pool would be polyglutamated and charged with one-carbon units *in situ*. Although folylpolyglutamates cannot be transported into the mitochondria, there is evidence that polyglutamates synthesized in this organelle are slowly released into the cytoplasm (Shane, 1995). On the other hand, glycine and serine are rapidly transportable across the mitochondrial membranes (Cybulski and Fisher, 1976), as is formate (Barlowe and Appling, 1988; Pasternack *et al*, 1992, and 1994a and b). Appling (1991) has emphasized the interdependence of these two compartments in yeast and higher animals. Therefore, mutants of CHO cells that are deficient in the mitochondrial form of SHMT are auxotrophic for glycine (Chasin *et al*, 1974). Clearly, the normal cytosolic SHMT activity of these cells is unable to satisfy its glycine requirement. In fact, overexpression of human SHMT activity in the cytosol of these cells does not overcome the glycine requirement. This study along with others (Lowe *et al*, 1993; Lin and Shane, 1994) using CHO cell mutants that lack

FPGS activity (AUX B1) have demonstrated that mitochondrial folate metabolism provides C_1 units for cytoplasmic metabolism.

1.3.1. Compartmentation of folate metabolism in higher plants

Recently, Neuberger *et al* (1996) and Rébeillé *et al* (1997) reported on the occurrence of mitochondrial enzymes for folate synthesis in pea leaves (*Pisum sativum* L. var. Douce Provence). Their data suggest that the mitochondria may be a major site for tetrahydrofolate polyglutamate and thymidylate synthesis. They also suggested that these leaf mitochondria may contain a major part of the cellular folate (Neuberger *et al*, 1996). Despite this, their method for determining folate content may be inaccurate. In this regard, the radioisotopic binding assay described by Waxman and Schreiber (1980), though useful in applications for determining serum and erythrocyte folate, has limited application to whole cell extracts. This is due to the fact that different radiobinding kits give varying results for monoglutamates, depending on the oxidation state and the nature of the one-carbon substituent (Shane *et al*, 1980). In addition, the degree of response to pteroylpolyglutamates increases as greater amounts of folate are assayed. This suggests that the radioassay may not be suitable for quantifying the complex mixtures of folate that occur in plant extracts. The claim (Neuberger *et al*, 1996) that approximately 50% of the total folate pool is mitochondrial may therefore be an overestimation. Similarly, these workers failed to detect a cytosolic folate pool in pea leaf extracts. This is surprising in light of

numerous microbiological assays of plant species in which folate derivatives have been identified in the cytosol, mitochondria, and chloroplasts (for review, see Cossins, 1984).

It is clear that the total folate pool of higher plants is affected by light (Spronk and Cossins, 1972). This study demonstrated that green radish cotyledons contained nearly double the amount of folate seen in etiolated tissues of the same age but a smaller proportion of methylated derivatives. As well, the folate content in radish cotyledons harvested in the dark and light phases of a 13:11 h light:dark cycle was significantly different. Light-harvested tissues had less folate, a smaller proportion of formyl-substituted coenzymes, and a greater unsubstituted $H_4PteGlu_n$ pool as compared to the dark-harvested tissue (Spronk and Cossins, 1972). As noted earlier, light also affects the levels of GDC and SHMT (Walker and Oliver, 1986; Turner *et al*, 1993) as well as the activities of C_1-THF synthase (Chen *et al*, 1997) in pea leaves. It is also possible that some of the 5,10-CH_2-$H_4PteGlu_n$ arising from the photorespiratory cleavage of glycine and serine may be utilized by the C_1-THF synthase activities present in leaf mitochondria. However, little information exists on this aspect of folate metabolism in plants.

1.3.2. The present study

The results of two recent studies (Neuburger *et al*, 1996; Rébeillé *et al*, 1997) suggest that the biosynthesis of folate in higher plant cells is localized mainly in the

mitochondria. Prior to the present study, the intracellular distribution of folate derivatives in pea leaves had yet to be studied in detail. In this study, the compartmentation of folate coenzymes in actively growing leaves of *Pisum sativum* has been examined. A microbiological assay of folate involving glycerol-cryoprotected bacteria and 96-well microtiter plates was used to examine the mitochondrial folate pool isolated from green leaves as well as from etiolated leaves. Other folate pools such those in the cytosol and chloroplasts were also examined. Considering the dynamic flow of carbon in the photorespiratory cycle, it is possible that the demand for one-carbon units and the cellular folate pool will increase during greening. The magnitude of this change and the subcellular distribution of folate derivatives within extracts of greening pea leaves were also investigated. Characterization of each folate pool was accomplished with ion-pair reverse phase high performance liquid chromatography.

MATERIALS AND METHODS

2.1. Materials

General chemicals, of the highest grade commercially available, were obtained from either Sigma-Aldrich Ltd. (Oakville, ON), Fisher Scientific Ltd. (Nepean, ON), or British Drug House (BDH) Inc. (Toronto, ON). More specialized reagents were supplied by the following sources. BSA (Fraction V), defatted BSA (prepared from Fraction V albumin), PteGlu, (6*R,S*)-H_4PteGlu, and (6*R,S*)-5-HCO-H_4PteGlu, were purchased from Sigma. H_2PteGlu, (6*R,S*)-5-CH_3-H_4PteGlu, (6*R,S*)-10-HCO-H_4PteGlu, and (6*R,S*)-5,10-CH_2-H_4PteGlu, were synthesized according to the methods outlined by Horne *et al* (1981a). Bio-Gel P-6DG and protein assay dye reagent concentrate were products of Bio-Rad Laboratories (Hercules, CA). Diethylaminoethyl (DEAE) cellulose (DE52) was obtained from Whatman Inc. (Fairfield, NJ). Flat bottom, 96-well cell culture cluster dishes (i.e., microtiter plates) were purchased from Costar Corp. (Cambridge, MA), no. 3599. The high performance liquid chromatography (HPLC) of monoglutamyl folates used tetrabutylammonium hydroxide (TBAOH) from Fisher and μBondapak C_{18} columns supplied by Waters Corp. (Mississauga, ON). Lyophilized cultures of *L. rhamnosus* and *P. acidilactici* were obtained from the American Type Culture Collection (Rockville, MD). Microbiological media, including Bacto Lactobacilli Broth AOAC, Bacto Folic Acid Casei Medium, Bacto Agar, and Bacto Yeast Extract, were from Difco Laboratories (Detroit, MI).

2.2. Plant Material

Pea (*Pisum sativum* L. cv. Homesteader) seeds were purchased from Apache Seeds Ltd. (Edmonton, AB). Plants were raised under controlled conditions (16 h days with a light intensity of 400 μmol m^{-2} s^{-1}, 25°C; 8 h nights, 20°C; 50% R.H.) for 14 d as described by Imeson *et al* (1990). Leaves of etiolated seedlings, germinated and grown for up to 12 d under the conditions described above but in darkness, were harvested in subdued green light (15 watt fluorescent bulb covered with three Roscolux color filters (#10 - medium yellow, #91 - primary green, and #395 - teal green, Rosco Laboratories Inc. (Stamford, CT)) which had a transmission spectral peak at 510 ± 30 nm). In other experiments, light-treated seedlings were first dark-grown for 9 d and subsequently subjected to continuous light with an intensity of 400 μmol m^{-2} s^{-1} for up to 72 h. Leaf extracts were prepared immediately after excision.

2.3. Organelle Isolation and Extraction of Folates

2.3.1. Whole-leaf extracts

Pteroylpolyglutamates were extracted from whole apical leaves according to the method of Spronk and Cossins (1972) with the following modifications. Samples of tissue were immersed in 10 volumes of Extraction Buffer containing 50 mM HEPES, 50 mM CHES, 2% (w/v) sodium ascorbate, and 0.2 M 2-mercaptoethanol, pH 7.85 (Wilson and Horne, 1984), for 10 min. at 100°C. After cooling in ice, the tissue was ground with a Ten Broeck tissue grinder. Cellular debris and insoluble

matter was removed by centrifugation at 20,000*g* for 10 min. (Beckman JA-20 rotor in a Beckman J2-21M/E centrifuge (Mississauga, ON)) and the pellet was washed twice with 2 volumes of Extraction Buffer. The supernatants were combined and stored under argon at -20°C. Parallel extractions were performed without the heat treatment. These extracts were assayed for protein by the method of Bradford (1976) using BSA as the standard.

2.3.2. Cytosolic and mitochondrial extracts

The method of Douce *et al* (1987) was followed without modification to isolate mitochondria that were purified on Percoll gradients. Samples from fractions generated by differential centrifugation, including the initial homogenate, 1,500*g* pellet and supernatant, and 12,000*g* supernatant which is representative of the cytosolic fraction (Douce *et al*, 1987), were immediately diluted with 0.2 volumes of 5-fold concentrated Extraction Buffer, i.e., 0.25 M HEPES, 0.25 M CHES, 10% (w/v) sodium ascorbate, and 1 M 2-mercaptoethanol, pH 7.85 (Horne *et al*, 1989). The gradient-purified mitochondria were subjected to various solubilization treatments after adding 0.2 volumes of 5-fold concentrated Extraction Buffer. These included the following methods employed by Clandinin and Cossins (1972) and Chen *et al* (1997): 1. no additional treatment, 2. sonication (2 pulses of 2 min. each at 4°C) with the output control set at 70 using a Cole-Parmer Ultrasonic Homogenizer, 4710 Series (Chicago, IL), 3. incubation with 0.2% (w/v) sodium deoxycholate for 1 h at

4°C, 4. incubation with 0.2% (w/v) sodium deoxycholate for 1 h at 4°C and sonication, 5. incubation with 0.1% (v/v) Triton X-100 for 1 h at 4°C, and 6. incubation with 0.1% (v/v) Triton X-100 for 1 h at 4°C and sonication. Samples of 100 µL from the resulting suspensions were removed for protein measurement. The rest of the suspension was incubated at 100°C for 10 min. and centrifuged at 20,000*g* for 10 min. to remove denatured protein. The pellet was washed twice with 2 mL of Extraction Buffer and the supernatants were combined and stored at -20°C under argon.

2.3.3. Chloroplastic extracts

Pea leaf chloroplasts, isolated and purified by the method of Schuler and Zielinski (1989), were lysed by resuspending the intact chloroplasts in 0.2 volumes of 5-fold concentrated Extraction Buffer and 0.1% (v/v) Triton X-100 at 4°C for 1 h. Aliquots of 100 µL of the suspension were used for protein determination and the remainder was treated for 10 min. at 100°C and centrifuged at 20,000*g* for 10 min. to remove denatured protein. The pellet was washed twice with 2 mL of Extraction Buffer and the supernatants were combined and stored at -20°C under argon.

2.3.4. Assay of enzyme activities

The biochemical purity of the subcellular fractions was assessed using assays of marker enzymes. Activities of alcohol dehydrogenase (EC 1.1.1.1) as a cytosolic

marker (Racker, 1955), succinate dehydrogenase (EC 1.3.99.1) as a mitochondrial marker (Cooper and Beevers, 1969), and phosphoribulokinase (EC 2.7.1.19) as a chloroplastic marker (Kagawa, 1982), were assayed.

Glycolate oxidase (EC 1.1.3.15) activity was measured in leaf extracts prepared from 9 and 11 d etiolated seedlings and 48 h light-treated seedlings by the method of Murray *et al* (1973).

2.4. Enzymatic Hydrolysis of Pteroylpolyglutamates

γ-Glutamyl hydrolase was prepared from 4 d pea cotyledons according to Lin *et al* (1993b). Protein precipitating in the 40-70% range with $(NH_4)_2SO_4$ was desalted by passage through Bio-Gel P-6DG and used at a concentration of 2 mg mL^{-1}. Pteroylpolyglutamates were hydrolyzed under argon in 4 mL reaction systems containing 1 mL of folate sample and 0.5 mL of GGH preparation, with 5% (w/v) sodium ascorbate and 75 mM sodium acetate buffer (pH 6.0), at 37°C with a final pH of 6.3. In preliminary work, this incubation was allowed to run for up to 24 h with samples taken at various intervals. The results from this time course study showed that incubations longer than 5 h produced insignificant increases in recovered folate and as such, future hydrolyses were stopped by incubation of each sample at -70°C after 5 h. A sample of Bacto Yeast Extract was routinely taken through the identical protocol to verify hydrolytic activity; a 10-fold increase in assayable folates after enzyme treatment of the yeast extract confirmed activity (Coffin, 1985).

2.5. Microbiological Assay of Folate Derivatives

2.5.1. Glycerol-cryoprotected bacteria

Analyses of folate were carried out in microtiter plates using glycerol-cryoprotected bacteria. Cultures of *L. rhamnosus* and *P. acidilactici* were prepared by the method of Horne and Patterson (1988) at 8 month intervals in order to maintain a high level of viability of the cryoprotected cells throughout assays (Grossowicz *et al*, 1981). Single strength Bacto Folic Acid Casei Medium was prepared by dissolving 9.4 g of dehydrated medium in 200 mL of distilled water. After sterilization at 121°C for 20 min., 50 mg of sodium ascorbate and 120 ng of (6*R*,*S*)-5-HCO-H_4PteGlu were added using a 0.22 μm pore size filter unit. The lyophilized culture (as received from the ATCC) was suspended in 10 mL of Bacto Lactobacilli Broth AOAC. After three successive transfers to fresh broth upon incubation at 37°C for approximately 24 h, 0.5 mL of this inoculum was added to the folate-supplemented assay medium and this mixture was incubated for approximately 18 h at 37°C. It was then cooled in an ice bath, mixed with an equal volume of 4°C, sterile 80% (v/v) glycerol, and stored in 2.5 mL aliquots at -70°C.

2.5.2. Maintenance of microorganisms

Stock cultures of the bacteria were kept in Bacto Lactobacilli Broth AOAC with 1% (w/v) Bacto Agar. These stab-inoculated agar slants were refrigerated after

18 h at 37°C and transferred to fresh maintenance medium at monthly intervals to preserve the viability and sensitivity of the assay organisms.

2.5.3. Assay procedure

The folate assay described by Horne and Patterson (1988) was followed with a few modifications. Bacto Folic Acid Casei Medium was prepared as described by the manufacturer's instructions. After suspending 9.4 g of powdered medium in 100 mL of distilled water, it was heated to boiling for 1 min. and autoclaved at 121°C for 20 min.

The inoculum was prepared by diluting the 2.5 mL aliquot of thawed bacterial suspension 3-fold with sterile 0.9% (w/v) sodium chloride solution. This was the highest dilution of the bacteria that gave the greatest absorbance for all points on the standard curve when compared to other dilutions of the inoculum.

Working buffer was prepared by dissolving 1.6 g of sodium ascorbate in 9.5 mL of distilled water, to which 0.5 mL of 1 M potassium phosphate buffer (pH 6.1) had been added, and sterilized by filtration.

The folate standard stock solution contained the calcium salt of (6*R,S*)-5-HCO-H_4PteGlu at a concentration of 2.5 mM. Folate concentration was determined by measuring A_{285} in appropriate dilutions with 0.1 M potassium phosphate (pH 7.0), using a molar extinction coefficient of 37.2 x 10^3 cm^{-1} M^{-1} (Uyeda and Rabinowitz, 1965). As only the (6*S*)-isomer supports growth of the assay organisms, the

concentration of the active isomer is half of that determined by absorption. The stock solution was stored under argon in aliquots of 100 µL at -70°C. The working standard was prepared by diluting it with the same diluent used for the samples (e.g., 1% (w/v) sodium ascorbate (pH 6.0) or HPLC reagent).

Working buffer (8 µL) was added to each well of a sterile 96-well microtiter plate. Wells for the standard curve contained, in triplicate, from 10 to 120 femtomoles of (6*S*)-5-HCO-H_4PteGlu per well in a maximum volume of 100 µL. The sample, diluted with 1% (w/v) sodium ascorbate (pH 6.0) if necessary, ranged up to 122 µL. To prevent folate contamination of the samples and standards, the next three steps are performed first to the blanks, then to the samples, followed by the remaining standards. The volume of the standards and samples was adjusted to 122 µL with the appropriate diluent. Also added to each well was 150 µL of Bacto Folic Acid Casei Medium and 20 µL of the inoculum. The polystyrene cover was placed on the microtiter plate and incubated for approximately 18 h. This was carried out in a small pan, containing the plate placed on top of water-soaked cheesecloth, wrapped with a plastic film (e.g., Saran Wrap) and placed in an incubator at 37°C. Contrary to Horne's finding (1997), it was found necessary to resuspend the contents of each well after the incubation period by repeatedly aspirating the samples using a pipette (Horne and Patterson, 1988). Bacterial growth was then measured by reading the turbidity of each well at 630 nm in a model EL311 Microplate Autoreader (Bio-Tek Instruments Inc., Winooski, VT) which was interfaced with an Apple Power Macintosh computer.

2.6. Separation of Pteroylmonoglutamate Derivatives by HPLC

Initially, the DEAE cellulose separation of folate coenzymes was performed by the method of Sotobayashi *et al* (1966). However, HPLC was found to be a superior method for separating and quantifying monoglutamate derivatives because the procedures involved in conventional ion-exchange chromatography suffer from a lack of reproducibility (e.g., variations in elution volumes and resolution due to re-packing of the column after each run) and sensitivity (e.g., inability to separate certain folates) as well as being time consuming (e.g., taking up to 10 times longer than HPLC to elute folates and regenerate a column prior to loading the next sample).

HPLC separation of folate derivatives was based on the procedures described by Wilson and Horne (1986). In the present studies, a Waters μBondapak C_{18} steel column (3.9 x 300 mm) was used with a Varian VISTA 54 solvent delivery system (Walnut Creek, CA) equipped with a Rheodyne model 7125 injector (Cotati, CA). Tetrabutylammonium phosphate (TBAP) reagent for paired-ion chromatography was prepared with TBAOH according to Horne *et al* (1981a). The mobile phase, consisting of 7 mM TBAP reagent, 1 mM sodium ascorbate, and 10% (v/v) ethanol, was isocratically pumped throughout the 72 min. run. This chromatographic program was run at a flow rate of 1 mL $min.^{-1}$ at 22°C following a 200 μL sample injection. Fractions of 1 mL were collected in the dark using a Redifrac fraction collector (Pharmacia Biotech Inc., Baie d'Urfe, QC) in the presence of 0.1 mL of 10% (w/v) sodium ascorbate (pH 6.0) to protect labile folates. The fractions were then

immediately subjected to microbiological assay. Basic criteria described by Sengupta and Cossins (1971) were used to positively identify the pteroylglutamate derivatives in fractions collected from the columns. These included comparing the retention time of the unknown derivative with that of authentic standards, the differential growth response of the two assay organisms, and co-chromatographing the extract with authentic folate derivatives.

RESULTS

Prior to the study of plant extracts, a number of procedures were optimized for folate analysis. These included the hydrolysis of conjugated derivatives, the solubilization of pteroylglutamate derivatives from organelles, the assay of total folate, and the chromatographic fractionation of the coenzyme forms of folate. Once this was achieved, these conditions were applied to the folate samples derived from pea leaves.

3.1. γ-Glutamyl Hydrolase Treatment of Folate Samples

Earlier studies have established that the folate pool of pea seedlings is highly conjugated (Imeson *et al*, 1990; Zheng *et al*, 1992). In fact, monoglutamate derivatives were not readily detected in 14 d pea leaves (Imeson *et al*, 1990). In the majority of analyses, the determination of the chain length of folylpolyglutamates was not required. This greatly simplifies the analysis by allowing a determination of the folate derivatives in their monoglutamyl forms after treatment with a γ-glutamyl carboxypeptidase. The quantitative microbiological assay of conjugated derivatives requires pre-treatment of the extracts with γ-glutamyl hydrolase. As noted earlier (see section 1.1.1), sample treatment with this enzyme is a critical step of the microbiological assay because the *L. rhamnosus* response to $PteGlu_{>3}$ is negligible and *P. acidilactici* does not respond to derivatives with more than two glutamate residues (Tamura, 1990). Hydrolases from a wide variety of sources have been used in this

laboratory including that from pea seeds (Roos and Cossins, 1971; Coffin and Cossins, 1986). Folate hydrolase treatment with this enzyme has routinely been carried out at 37°C for 2 h at pH 4.5 (Roos and Cossins, 1971; Coffin and Cossins, 1986). However this endopeptidase reaction, which yields pteroylmonoglutamates, occurs maximally at pH 6.0, with 50% of maximum detected at pH 4.5 and 7.2 (Lin *et al*, 1993b). When mitochondrial folates from 14 d pea leaves were hydrolyzed with this enzyme at pH 6.3 for different lengths of time (see section 2.4), hydrolysis was not complete after 3 h (Figure 5). Incubations up to 8 h produced increases in recovered folate before net losses of folate were encountered. This is probably due to the labile nature of the folate molecule (Cooper *et al*, 1978). As the 14 d mitochondrial folate extracts represent the most concentrated folate samples examined, all subsequent samples were routinely terminated after 5 h of incubation with the hydrolase. The amount of folate from individual pea cotyledon GGH preparations was estimated using microbiological analysis. Quantities ranging from 100-200 fmol mL^{-1} were routinely subtracted from the values obtained from the hydrolyzed samples.

3.2. Release of Folates from Mitochondria and Chloroplasts

Pea leaf mitochondria (Douce *et al*, 1987) and chloroplasts (Schuler and Zielinski, 1989) were isolated using Percoll gradients. Mitochondrial membranes were disrupted by one of six treatments in addition to osmotic shock (see section

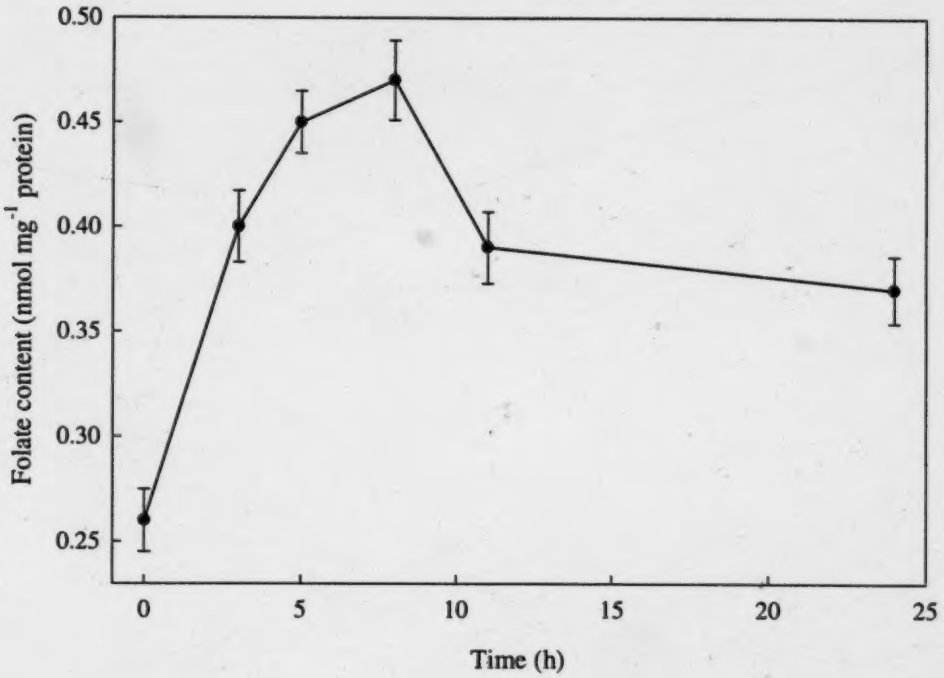


Figure 5. *The Hydrolysis of Folylpolyglutamates as a Function of Incubation Time.* Polyglutamyl folates present in 14 d pea leaf mitochondria were treated with pea cotyledon γ-glutamyl hydrolase for the times indicated, removed from the suspension, incubated at 100°C for 2 min., and then assayed for folate using glycerol-cryoprotected *L. rhamnosus*. Values are reported as means ± SEM, n = 3.

2.3.2) and assayed for folate before and after treatment with γ-glutamyl hydrolase (Table 2). While Clandinin and Cossins (1972) reported that sodium deoxycholate treatment together with either osmotic shock or sonication was the most effective solubilizing treatment, this was not apparent with the six treatments used in the present study. These six treatments appeared to be equally effective in releasing folate from isolated mitochondria (Table 2). This may be because the solubilization step was allowed to occur over a longer period of time (i.e., 1 h as opposed to 30 min.). More probable though, the incubation of the isolated organelle at 100°C for 10 min., which was not carried out in this earlier study (Clandinin and Cossins, 1972), released membrane- and protein-bound folates. It is noteworthy that studies of mammalian tissues only employ this latter step to solubilize folate from mitochondria (Horne *et al*, 1989; Balaghi *et al*, 1993; Horne and Holloway, 1997). As no one method appeared superior, subsequent folate extracts from either mitochondria or chloroplasts involved osmotic shock and 0.1% (v/v) Triton X-100 treatments. Besides being effective in releasing tightly bound folates, this detergent at a concentration of 0.1% (v/v) did not appear to significantly affect the microbiological assay (Table 2).

3.3. The Assay of Folate Derivatives

While new approaches to measure folate are available, the microbiological assay remains the method of choice (Tamura, 1990). This is because methods such as

Table 2. *Solubilization of Pteroylglutamate Derivatives from Isolated Mitochondria.*

Treatment	Before GGH	After GGH
1. No additional treatment	0.25 ± 0.03	0.48 ± 0.03
2. Sonication	0.24 ± 0.02	0.48 ± 0.04
3. 0.2% Na deoxycholate	0.26 ± 0.04	0.45 ± 0.03
4. 0.2% Na deoxycholate and sonication	0.23 ± 0.03	0.47 ± 0.03
5. 0.1% Triton X-100	0.27 ± 0.02	0.46 ± 0.03
6. 0.1% Triton X-100 and sonication	0.26 ± 0.02	0.45 ± 0.03

Mitochondria purified using Percoll gradients were solubilized by dilution with Extraction Buffer and subjected to various treatments (see section 2.3.2). Data are expressed in nmoles folate mg^{-1} protein, means ± SEM, n = 3.

colorimetry, fluorometry, radiobinding assay, radioenzymatic assay, and radioimmunoassay, are generally nonspecific and less sensitive. Therefore these methods appear to be less reliable than microbiological assays for quantifying complex mixtures of folate (Tamura, 1990). The use of glycerol-cryoprotected bacteria and 96-well microtiter plates, as shown in Figure 6, are two recent developments which offer even greater advantages to the standard bioassay. This alternative to the conventional microbiological assay shortens assay time, shows considerably less growth for the zero-folate blanks, and by detecting as little as 10 fmol of folate, is over 10-fold more sensitive. A typical standard curve generated using this assay is shown in Figure 7. Assays were routinely very straightforward and replicates were in close agreement (i.e., a SEM of less than 5%).

The "edge effect" observed by Horne (1997), whereby the outside perimeter wells of the microtiter plates (i.e., rows A and H, and columns 1 and 12) gave statistically-significantly higher readings than identical samples on the interior, was only evident in the present system when samples collected during HPLC were assayed. In these cases, the outside wells were routinely not used.

3.4. The Separation of Pteroylmonoglutamate Derivatives

Many different methods exist to separate individual folate derivatives. These include bioautography, electrophoresis, gel filtration, and ion exchange-, thin layer-, and high performance liquid chromatography (Blakley, 1969; Cossins, 1984; Cossins

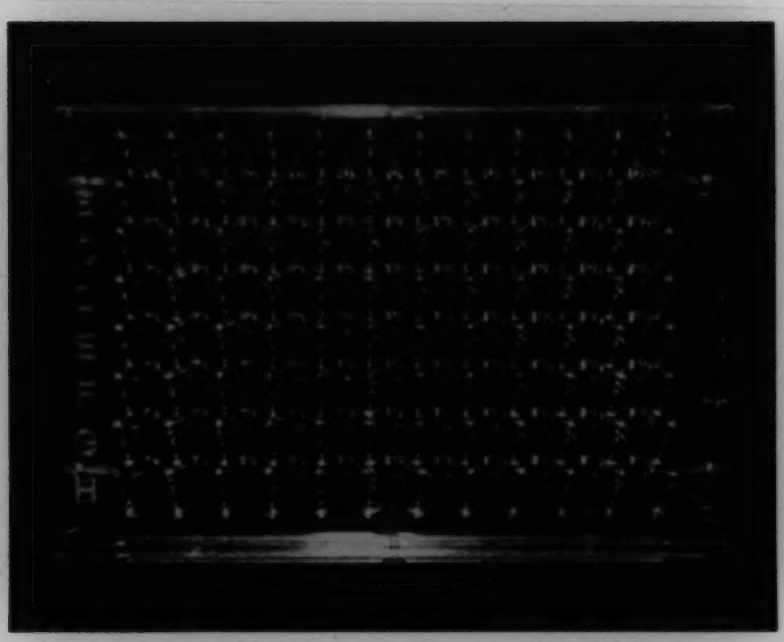


Figure 6. *The Microbiological Assay of Folate Using 96-Well Microtiter Plates.* A picture of a typical microtiter plate used for the microbiological assay of folate derivatives. The first three rows (A-C) are used to generate the standard curve while the remaining wells are used to assay samples.

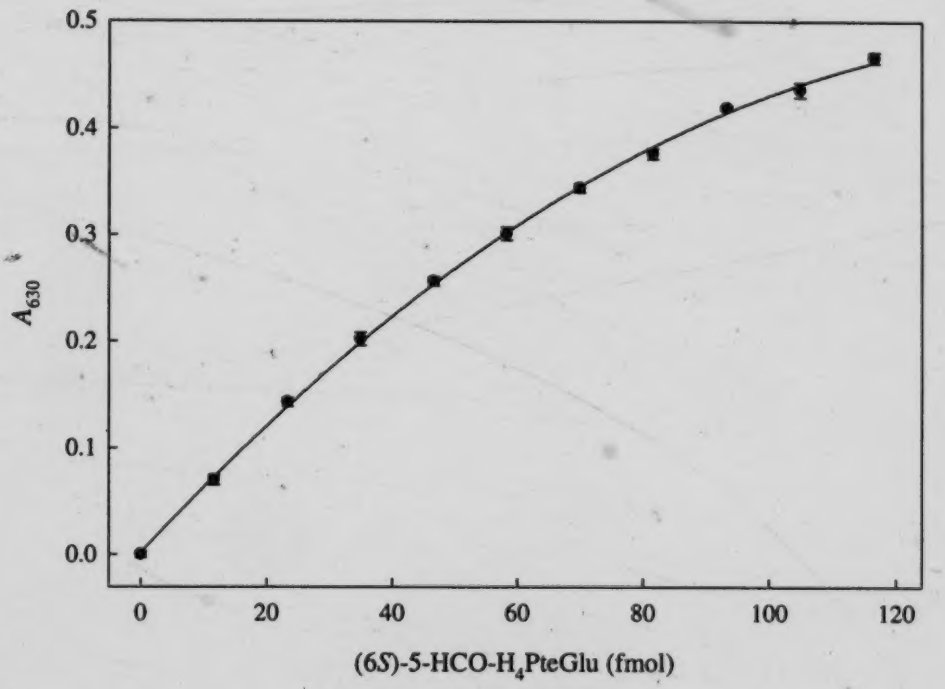


Figure 7. *Standard Curve for the Microbiological Assay of Folate.*

The growth response of glycerol-cryoprotected *L. rhamnosus* to varying amounts of 5-HCO-H_4PteGlu using 96-well microtiter plates.

and Chen, 1997). However, their usefulness in the study of plant folate pools is often precluded by their relatively high detection limit. Ion-pair, reverse phase HPLC, when used in conjunction with the microbiological assay, is however a viable method for examining the folate pools of plant tissues.

Standard solutions of authentic folates were initially separated using an isocratic program containing ethanol. These separations were used to obtain retention times for a wide range of biologically important derivatives (Figure 8). Combined with the differential microbiological assay, this allowed for the positive identification of peaks obtained when leaf extracts were injected. This chromatographic program did not employ the ethanol gradient used by Wilson and Horne (1986) as it was clear that the growth responses of *L. rhamnosus* and *P. acidilactici* are inhibited by this reagent. Nevertheless, seven different folates were essentially baseline separated and eluted in the same order as that reported by Wilson and Horne (1986). Recoveries of 5-HCO-H_4PteGlu and PteGlu from the column ranged from 90 to 105% of the amount injected.

3.5. The Folate Content of Pea Leaves

3.5.1. Folate content as a function of leaf age

Earlier work has established that the folate status of plant seedlings changes during germination and early seedling growth (Roos *et al*, 1968; Roos and Cossins, 1971; Spronk and Cossins, 1972). In this regard, pteroylglutamate levels are very

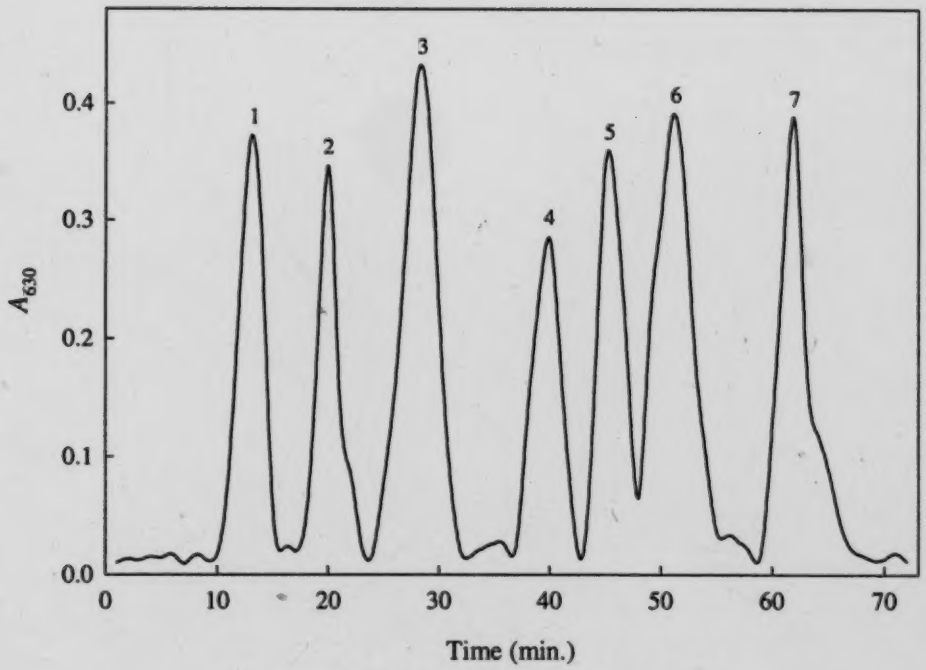


Figure 8. *The Separation of Folate Derivatives by HPLC and* L. rhamnosus *Assay.* The elution of seven folate derivatives with 10% (v/v) ethanol as detected by *L. rhamnosus*. The derivatives are: 1. 10-HCO-H_4PteGlu (13 min.), 2. H_4PteGlu (20 min.), 3. 5-HCO-H_4PteGlu (28 min.), 4. H_2PteGlu (40 min.), 5. 5,10-CH_2-H_4PteGlu (45 min.), 6. 5-CH_3-H_4PteGlu (51 min.), and 7. PteGlu (62 min.). Between 20-25 pmol of each derivative were applied to the column.

low in pea and radish cotyledons after 24 h of imbibition but show dramatic increases with maximum levels being reached by 6 to 7 d before declining in older tissues (Roos and Cossins, 1971; Spronk and Cossins, 1972). Examination of 9 to 14 d pea leaf extracts suggested that this dynamic pool also exists in leaves (Table 3). In agreement with earlier work (Cossins and Shah, 1972), 14 d whole-leaf extracts were found to have a total folate content of 4.72 ± 0.10 nmol g^{-1} fresh weight. In addition, folate content tended to decrease with increasing age, a phenomenon also observed in the radicles of *Pisum sativum* (Sengupta and Cossins, 1971). Thus, the 14 d extracts were found to contain less folate than the 9 and 11 d samples. On a protein basis, the folate levels in 14 d extracts were approximately half of that found in extracts of leaves that were 5 d younger (Table 3).

The older tissues also had a greater proportion of short-chain folates as determined before and after treatment with folate hydrolase (Table 4). Over half of the pteroylglutamate pool was not detected by *L. rhamnosus* before GGH treatment in all three extracts. However, increases in bacterial growth seen after hydrolysis of the polyglutamate chain ranged from 113% for the 14 d leaf extract to 143% for the 9 d extract, a range similar to that reported by Spronk and Cossins (1972).

While the amount of folate changed with leaf age, the distribution of folate coenzymes was little affected (Table 3). The 5-CH_3-H_4PteGlu and H_4PteGlu derivatives represented the bulk of the folates in all three extracts with smaller levels of 5-HCO-H_4PteGlu also detected. While the proportion of the methylated pool is in

Table 3. *The Folate Content of 9 to 14 d Pea Leaves.*

Pea leaves were extracted and assayed for total folate using *L. rhamnosus* (see "Materials and Methods"). The distribution of individual folate derivatives was determined after HPLC and microbiological assay of column effluents. Values are reported as means ± SEM, n = 3.

Age (d)	Total folate content		Distribution of major H_4PteGlu derivatives (%)		
	nmol g^{-1} f.wt.	nmol mg^{-1} protein	Unsubstituted	5-formyl	5-methyl
9	5.22 ± 0.31	0.30 ± 0.02	34.8 ± 2.6	9.0 ± 0.6	56.4 ± 3.2
11	5.23 ± 0.24	0.18 ± 0.01	30.6 ± 0.3	8.2 ± 0.5	61.4 ± 0.8
14	4.72 ± 0.10	0.17 ± 0.01	36.3 ± 2.3	9.1 ± 0.2	54.7 ± 2.3

Table 4. *Proportion of Total Folate Detected Before GGH Treatment in 9 to 14 d Pea Leaves.*

Pea leaves were extracted and assayed for folate, prior to treatment with γ-glutamyl hydrolase, using *L. rhamnosus* (see "Materials and Methods"). Values are reported as means ± SEM, n = 3.

Age (d)	Folate content		% of Total folate
	nmol g^{-1} f.wt.	nmol mg^{-1} protein	
9	2.15 ± 0.11	0.12 ± 0.01	40.8 ± 0.4
11	2.27 ± 0.16	0.08 ± 0.01	43.4 ± 0.4
14	2.22 ± 0.20	0.08 ± 0.01	46.9 ± 0.1

agreement with earlier studies (Cossins and Shah, 1972; Spronk and Cossins, 1972), the absence of 10-HCO-H_4PteGlu is surprising in light of its role in purine biosynthesis and in the initiation of organellar protein synthesis (see sections 1.2.4 and 1.2.7). Also, earlier studies have demonstrated that this formyl-substituted derivative is present in significant amounts of pea leaves and chloroplasts (Cossins and Shah, 1972; Spronk and Cossins, 1972), and pea cotyledon mitochondria (Clandinin and Cossins, 1972). Possible reasons for the failure to detect this formylated derivative are considered in the Discussion (see section 4.1.2).

3.5.2. Intracellular distribution of folate in 14 d pea leaves

Pteroylglutamates are known to exist in the cytosol, mitochondria, and chloroplasts of plant cells (for review, see Cossins, 1984). To determine the proportion of total leaf folate associated with the mitochondria, leaf extracts were prepared in the presence of an osmoticum to facilitate isolation of intact mitochondria (Douce *et al*, 1987). This procedure resulted in the recovery of 74% less total folate (1.23 ± 0.11 nmol g^{-1} fresh weight) possibly due to the less rigorous grinding procedure (Table 5). This procedure was however successful in isolating a low yield of mitochondria that were free of the cytosolic marker enzyme, alcohol dehydrogenase, and the chloroplastic marker enzyme, phosphoribulokinase (Table 6). Differential centrifugation of these extracts (Table 5) showed that folate derivatives were principally associated with the cytosolic fraction (i.e., 12,000*g* supernatant)

Table 5. *The Intracellular Distribution of Folate Derivatives in Leaves of 14 d Pea Seedlings.*

Pea leaves were extracted under isotonic conditions, fractionated by differential centrifugation, and assayed for total folate using *L. rhamnosus* (see "Materials and Methods"). The distribution of individual folate derivatives was determined after HPLC and microbiological assay of column effluents. Values are reported as means ± SEM, n = 7 for folate content and n = 4 for distribution.

Fraction	Total folate content		Distribution of major H_4PteGlu derivatives (%)			
	nmol 100 g^{-1} f.wt.	nmol mg^{-1} protein	10-formyl	Unsubstituted	5-formyl	5-methyl
Homogenate [a]	123.4 ± 10.7	0.12 ± 0.01	n.d. [b]	42.5 ± 0.2	5.8 ± 0.4	51.8 ± 0.2
1,500*g* Pellet [c]	19.2 ± 1.2	0.28 ± 0.02	9.0 ± 1.2	54.0 ± 2.9	25.7 ± 3.3	11.2 ± 0.8
1,500*g* Supernatant	99.3 ± 9.5	0.12 ± 0.01	n.a. [d]	n.a.	n.a.	n.a.
12,000*g* Supernatant	84.9 ± 8.2	0.11 ± 0.01	12.2 ± 5.4	22.1 ± 6.6	n.d.	65.8 ± 1.3
Purified mitochondria	0.9 ± 0.2	0.47 ± 0.11	3.2 ± 0.2	40.3 ± 0.1	53.5 ± 0.1	3.1 ± 0.3

[a] Initial homogenate was filtered, incubated at 100°C with Extraction Buffer, and centrifuged to remove denatured protein. [b] Not detected. [c] This pellet contained chloroplasts, nuclei, and intact cells. [d] Not assayed.

Table 6. *The Biochemical Purity of Subcellular Fractions Collected During Mitochondrial Isolation.*

Pea leaves were extracted under isotonic conditions, fractionated by differential centrifugation, treated with 0.1% (v/v) Triton X-100, and assayed for marker enzyme activities (see "Materials and Methods"). Data are expressed in percentages of total enzyme activity recovered, means ± SEM, n = 12.

Fraction	Alcohol dehydrogenase	Succinate dehydrogenase	Phosphoribulokinase
Homogenate	100	100	100
1,500*g* Pellet	5 ± 0.3	89 ± 4.3	84 ± 4.1
1,500*g* Supernatant	95 ± 3.4	11 ± 0.5	16 ± 1.0
12,000*g* Supernatant	93 ± 3.7	0.1 ± 0.01	10 ± 0.5
Purified mitochondria	n.d. [a]	7 ± 0.4	n.d.

[a] Not detected.

which was mostly devoid of contamination from mitochondria and chloroplasts (Table 6). This folate pool, like that of rat liver cytosol (Horne *et al*, 1989), was composed mainly of 5-CH_3-H_4PteGlu, with smaller amounts of unsubstituted folate plus 10-HCO-H_4PteGlu. Failure to detect 5-HCO-H_4PteGlu in the cytosol was unexpected as this derivative is present in the initial homogenate and approximately 70% of the cellular folate pool is located in the cytosol (Table 5). The mitochondrial fraction contained a much smaller proportion of the cellular folate pool and, in agreement with an earlier study (Besson *et al*, 1993), these were highly variable between individual extracts. HPLC analyses showed that the mitochondrial folate pool was composed mostly of the 5-HCO-H_4PteGlu and H_4PteGlu derivatives like that of rat pancreas mitochondria (Horne and Holloway, 1997). However, pea leaf mitochondria are known to partially co-sediment with chloroplasts even during low-speed centrifugation (Besson *et al*, 1995). Based on the recovery of succinate dehydrogenase, the yield of mitochondria was approximately 7% in the present study (Table 6). Therefore, the total mitochondrial fraction may in fact contain approximately 13.6 ± 3.0 nmol folate 100 g^{-1} fresh weight or 11.0 ± 2.3% of the cellular folate pool, a slightly higher value than that reported for pea cotyledons (Clandinin and Cossins, 1972).

In order to study the chloroplastic folate pool, pea leaves were gently homogenized in isotonic medium to isolate intact chloroplasts. This was successful in recovering 8.2 ± 0.5% of these organelles, as judged by measuring recovered

phosphoribulokinase activity. These purified chloroplasts exhibited negligible alcohol and succinate dehydrogenase activities. However when examined for total folate content, they were found to contain a considerable share of the cellular folate pool though it is much less concentrated in these organelles (0.02 ± 0.001 nmol folate mg^{-1} protein) as compared to mitochondria (0.47 ± 0.11 nmol folate mg^{-1} protein). This pattern has also been observed in an earlier study (Neuburger *et al*, 1996) where, on a protein basis, mitochondrial folate was 160 times more concentrated as these workers only detected 2.5 ± 0.5 pmol folate mg^{-1} chloroplast protein. These two organelles contain a fairly similar sized folate pool as the chloroplastic pool represented $8.4 \pm 0.6\%$ of cellular folate, a value in agreement with Neuburger *et al* (1996). Unfortunately due to the low concentration of pteroylglutamate in chloroplasts, HPLC combined with the microbiological assay failed to characterize this folate pool in greater detail. However, only $71.8 \pm 4.3\%$ of the chloroplastic pool supported *P. acidilactici* growth. This indicates that 5-CH_3-H_4PteGlu and/or oxidized PteGlu contribute significantly to this pool. The stripped thylakoids collected from the Percoll gradient used to purify the intact plastids were chromatographed and found to contain principally 5-HCO-H_4PteGlu ($60.7 \pm 1.6\%$) and H_4PteGlu ($32.2 \pm 1.3\%$), with smaller amounts of 5-CH_3-H_4PteGlu ($7.1 \pm 0.3\%$). As the 1,500*g* pellet from the mitochondrial isolation is rich in chloroplasts (Table 6), along with mitochondria, nuclei, and intact cells, this fraction was also examined for folate content and found to be highly concentrated with folate. In addition, the folate from this pellet was

composed primarily of unsubstituted H_4PteGlu and 5-HCO-H_4PteGlu (Table 5). Surprisingly, neither of the two latter folate extracts with chloroplastic content had a similar profile to that of an earlier study where the 5-CH_3-, 10-HCO-, and 5-HCO-H_4PteGlu coenzymes were equally represented in pea chloroplasts (Cossins and Shah, 1972).

3.6. The Effect of Greening on Folate Pools

3.6.1. Folate content of etiolated and greening pea leaves

Five folate-dependent enzyme activities, GDC, SHMT, DHY, CYC, and SYN, are affected during the development of photorespiration in greening tissues (Walker and Oliver, 1986; Turner *et al*, 1993; Chen *et al*, 1997). Therefore, it was of interest to examine the effect of greening on the levels of pteroylglutamates. To this end, pea seedlings were grown in the dark for 9 d and either kept in the dark for another 72 h or subjected to continuous exposure of light for the same length of time (Figure 9). When 9 d etiolated pea seedlings were subjected to continuous light for 72 h, the amount of folate increased while the etiolated seedlings saw little change (Figure 10). Maximal levels were reached by 48 h, at which time the etiolated tissues contained 60-70% of the folate content in green tissues of the same age. During this same period of time, the specific activity of glycolate oxidase increased nearly 3-fold. This is indicative of the onset of photorespiration as glycolate oxidase activity increased from 4.8 ± 0.2 to 13.9 ± 1.0 nmol $min.^{-1}$ mg^{-1} protein.

A B C

Figure 9. *Effect of Light on 9 d Etiolated Pea Seedlings.*

A picture of: A. 9 d etiolated pea seedlings, B. 11 d etiolated pea seedlings, and C. 9 d etiolated pea seedlings that were treated with continuous light for 48 h. These seedlings were harvested by excising the hypocotyls at the vermiculite level.

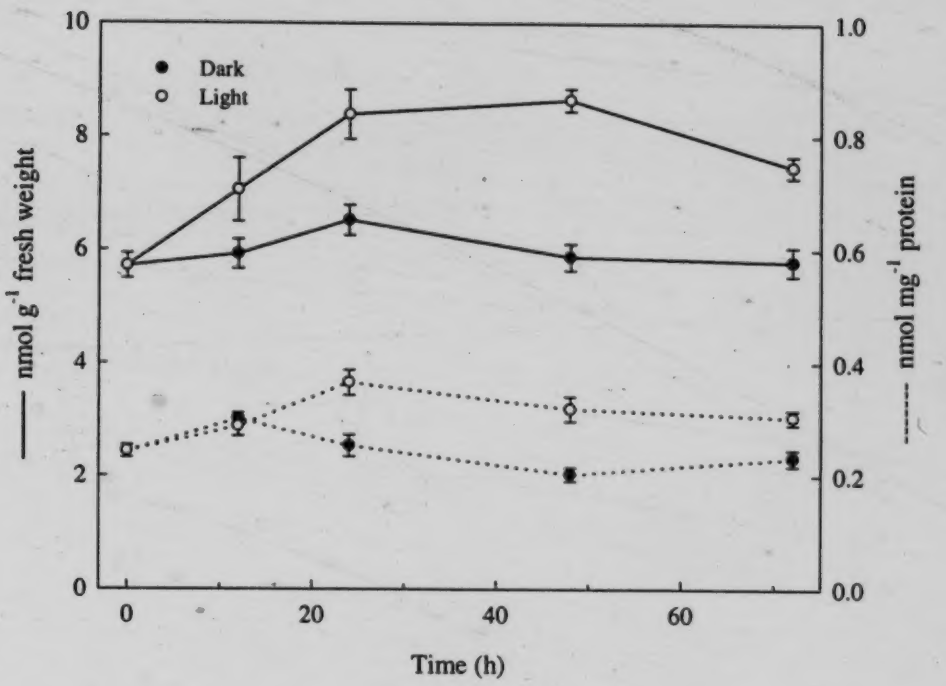


Figure 10. *Folate Content of Etiolated and Light-treated Pea Seedlings.*

The total folate content of pea leaves on the basis of fresh weight and protein when 9 d etiolated seedlings were kept in the dark or exposed to continuous light for 72 h. Data are expressed as means ± SEM, n = 9.

The degree of glutamyl conjugation of these folates was apparently different when extracts of etiolated and greened tissues were compared. The dark controls had a greater proportion of longer chain folates (i.e., folate derivatives with a glutamate chain length of more than 3 residues) than those that were treated with light (Figure 11). As a result, approximately 15% of the total folate pool supported *L. rhamnosus* growth prior to GGH treatment throughout the 72 h dark experimental period. In contrast, with increasing lengths of the light treatment, the folate pool became progressively less conjugated. Thus, nearly half of the cellular pool was readily assayable by microbiological means without the use of folate hydrolase by 48 h (Figure 11).

The distribution of individual folate coenzymes in whole leaves was also affected by greening (Figure 12). In general, the quantities of individual derivatives were lower in the etiolated tissues. However, the percentage of methylated compounds was slightly higher because the 48 h light treatment of 9 d pea seedlings increased the proportion of H_4PteGlu and 5-HCO-H_4PteGlu at the apparent expense of the 5-CH_3-H_4PteGlu pool, as had been previously documented (Spronk and Cossins, 1972). Therefore, while the amounts of the former two coenzymes each increased approximately 2-fold, the 5-CH_3-H_4PteGlu pool was only enlarged by a third. The presence of 10-HCO-H_4PteGlu in these extracts appeared to be variable and is discussed later (see section 4.1.2).

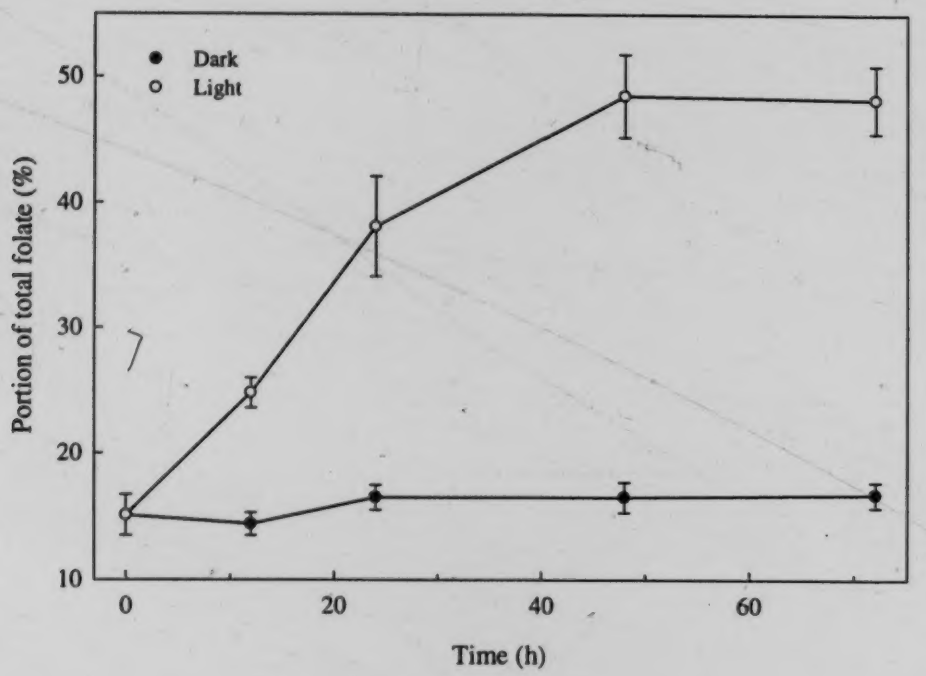


Figure 11. *Degree of Conjugation of Folate Derivatives in Greening Experiments.* The percentage of total cellular folate with three or less glutamate residues from etiolated and light-treated pea leaves as determined by *L. rhamnosus* assay before and after γ-glutamyl hydrolase treatment. Data are expressed as means ± SEM, n = 9.

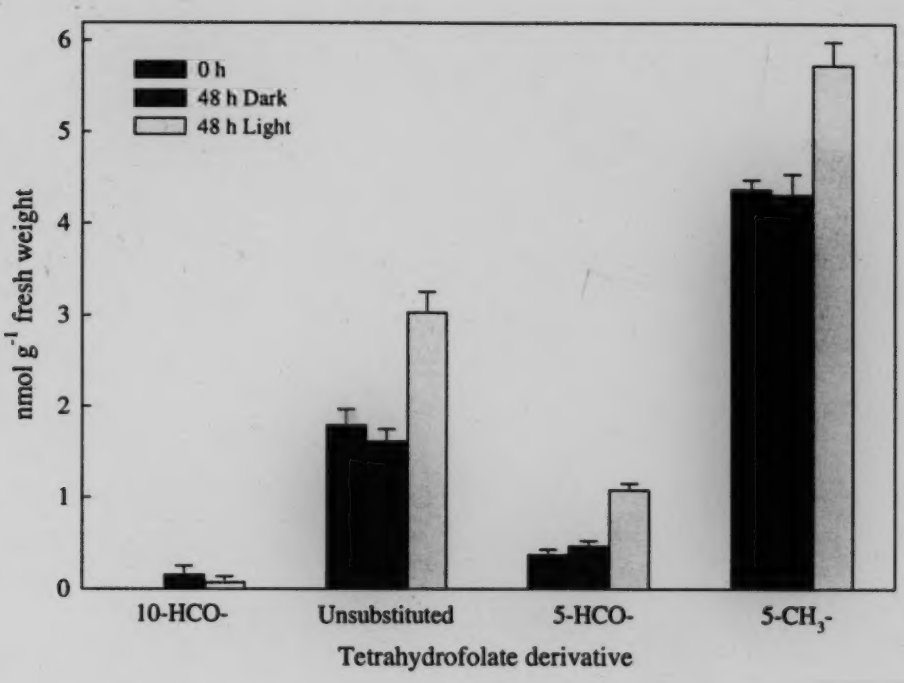


Figure 12. *The Distribution of Individual Folate Derivatives in Whole-leaf Extracts.* Folate from etiolated and greening leaf extracts were chromatographed by HPLC and quantified by microbiological assay of column effluent. Data are expressed as means ± SEM, n = 6.

3.6.2. Changes in the intracellular distribution of folate during greening

The mitochondrial activities of GDC and SHMT increase in greening tissues (for review, see Cossins and Chen, 1997). Therefore, it would be of interest to examine how the subcellular folylglutamate pools might be affected by greening. When isotonic conditions were employed to isolate intact mitochondria from etiolated and greening leaves, dramatic differences were observed between treatments (Tables 7-9). This includes a decrease in the cytosolic folate accompanied by an increase in the mitochondrial content. Therefore, the cytosolic compartment is responsible for approximately 85% of total folate in both etiolated samples (Tables 7 and 8). This is decreased by approximately 15% with light treatment as the mitochondrial folate pool size increased 10-fold (Table 9). Even with this dynamic change, mitochondria contain a mere 1.0 ± 0.2% of the cellular folate pool in light-treated leaves after adjusting for its recovery (Table 6).

In conjunction with the changes observed in the cellular pool sizes, the mitochondrial folate pool experienced a major transformation in the derivatives present. As both the 9 and 11 d mitochondrial extracts from etiolated seedlings were too dilute to chromatograph, these extracts were further characterized by assaying with *P. acidilactici*. Both extracts contained a large proportion of methylated derivatives and/or oxidized PteGlu as only 48.9 ± 4.1% and 52.1 ± 3.4% of the total mitochondrial pool was detected with this assay organism for the 9 and 11 d extracts,

Table 7. *The Intracellular Distribution of Folate Derivatives in Leaves of 9 d Etiolated Pea Seedlings.*

Pea leaves were extracted under isotonic conditions, fractionated by differential centrifugation, and assayed for total folate using *L. rhamnosus* (see "Materials and Methods"). The distribution of individual folate derivatives was determined after HPLC and microbiological assay of column effluents. Values are reported as means ± SEM, n = 3.

Fraction	Total folate content		Distribution of major H_4PteGlu derivatives (%)		
	nmol 100 g^{-1} f.wt.	nmol mg^{-1} protein	Unsubstituted	5-formyl	5-methyl
Homogenate [a]	96.84 ± 9.09	0.14 ± 0.001	44.9 ± 3.8	2.1 ± 0.4	53.1 ± 4.3
1,500*g* Supernatant [b]	89.00 ± 9.44	0.13 ± 0.01	n.a. [c]	n.a.	n.a.
12,000*g* Supernatant	81.14 ± 8.77	0.13 ± 0.01	44.5 ± 3.1	n.d. [d]	55.6 ± 3.2
Purified mitochondria	0.01 ± 0.002	0.01 ± 0.001	n.d.	n.d.	n.d.

[a] Initial homogenate was filtered, incubated at 100°C with Extraction Buffer, and centrifuged to remove denatured protein. [b] The pellet from this step contained chloroplasts, nuclei, intact cells, and detectable levels of *L. rhamnosus*-active folates which were not quantified. [c] Not assayed. [d] Not detected.

Table 8. *The Intracellular Distribution of Folate Derivatives in Leaves of 11 d Etiolated Pea Seedlings.*

Pea leaves were extracted under isotonic conditions, fractionated by differential centrifugation, and assayed for total folate using *L. rhamnosus* (see "Materials and Methods"). The distribution of individual folate derivatives was determined after HPLC and microbiological assay of column effluents. Values are reported as means ± SEM, n = 3.

Fraction	Total folate content		Distribution of major H_4PteGlu derivatives (%)		
	nmol 100 g^{-1} f.wt.	nmol mg^{-1} protein	Unsubstituted	5-formyl	5-methyl
Homogenate [a]	81.69 ± 5.34	0.13± 0.003	45.8 ± 2.2	2.8 ± 0.3	51.3 ± 1.9
1,500*g* Supernatant [b]	77.48 ± 5.51	0.13 ± 0.001	n.a. [c]	n.a.	n.a.
12,000*g* Supernatant	69.50 ± 5.49	0.11 ± 0.01	51.7 ± 1.4	n.d. [d]	48.4 ± 1.4
Purified mitochondria	0.01 ± 0.001	0.04 ± 0.01	n.d.	n.d.	n.d.

[a] Initial homogenate was filtered, incubated at 100°C with Extraction Buffer, and centrifuged to remove denatured protein. [b] The pellet from this step contained chloroplasts, nuclei, intact cells, and detectable levels of *L. rhamnosus*-active folates which were not quantified. [c] Not assayed. [d] Not detected.

Table 9. *The Intracellular Distribution of Folate Derivatives in Leaves of 9 d Etiolated Pea Seedlings Treated with 48 h of Light.* Pea leaves were extracted under isotonic conditions, fractionated by differential centrifugation, and assayed for total folate using *L. rhamnosus* (see "Materials and Methods"). The distribution of individual folate derivatives was determined after HPLC and microbiological assay of column effluents. Values are reported as means ± SEM, n = 3.

Fraction	Total folate content		Distribution of major H_4PteGlu derivatives (%)		
	nmol 100 g^{-1} f.wt.	nmol mg^{-1} protein	Unsubstituted	5-formyl	5-methyl
Homogenate [a]	157.71 ± 21.72	0.19 ± 0.03	40.4 ± 1.2	6.0 ± 0.7	53.6 ± 0.6
1,500*g* Supernatant [b]	135.38 ± 18.84	0.18 ± 0.03	n.a. [c]	n.a.	n.a.
12,000*g* Supernatant	113.47 ± 15.08	0.15 ± 0.02	36.3 ± 1.7	n.d. [d]	63.7 ± 1.7
Purified mitochondria	0.12 ± 0.04	0.11 ± 0.03	36.6 ± 1.1	56.2 ± 3.4	7.3 ± 1.6

[a] Initial homogenate was filtered, incubated at 100°C with Extraction Buffer, and centrifuged to remove denatured protein. [b] The pellet from this step contained chloroplasts, nuclei, intact cells, and detectable levels of *L. rhamnosus*-active folates which were not quantified. [c] Not assayed. [d] Not detected.

respectively. On the other hand, 5-HCO-H_4PteGlu and H_4PteGlu compose greater than 90% of the folate in light-treated plant mitochondria (Table 9). Interestingly, the former derivative was absent from the cytosolic fraction of the greening experiments, a phenomenon also observed in the mitochondrial isolation of 14 d leaves (see section 3.5.2). As experienced with earlier extracts, problems were encountered in detecting 10-HCO-H_4PteGlu. This is discussed in the following section.

DISCUSSION

The topics presented in the previous section will now be discussed. The methods employed in the present study are examined, comparisons between plant and mammalian folate content are drawn, and conclusions are made as to the nature of the pteroylglutamates present in *Pisum sativum* and the possible roles that they perform. In addition, suggestions are made for future studies that could provide further new information on the folate status of higher plants.

4.1. Evaluation of the Methods Employed in the Present Study

4.1.1. Microbiological assay of folates in microtiter plates

The "edge effect" (see section 3.3) described by Horne (1997) was only evident in the present studies when fractions collected during HPLC were assayed. This may be associated with the presence of ethanol in the samples which may partially evaporate during the incubation period. This would effectively concentrate the bacterial cells in a smaller volume resulting in a higher absorbance. The fact that this was not observed during assay of extracts without ethanol supports this possibility. As well, the present studies used different microtiter plates (Costar vs. Microtest III, Falcon no. 3072), microplate readers (Bio-Tek vs. Bio-Rad model 2550 EIA), and incubation conditions (microtiter plate placed in a sealed pan at 37°C vs. a humidified oven), than specified in Horne's study. Furthermore, failure to resuspend the assay organisms prior to measuring the absorbance, may have contributed to the

higher readings observed in the outer perimeter wells. The use of black-wall plates has been recommended to eliminate this problem (Horne, 1997).

4.1.2. Hydrolysis of pteroylpolyglutamates

The inconsistent detection of 10-HCO-H_4PteGlu in the present work was surprising as other workers have reported the presence of this folate in several higher plant species (Shah and Cossins, 1970; Roos and Cossins, 1971; Clandinin and Cossins, 1972; Cossins and Shah, 1972; Spronk and Cossins, 1972). It should be noted however, that because solutions of ascorbic acid were used to extract pteroylglutamates in these early studies, chemical conversions between some tetrahydrofolate compounds could have occurred (Wilson and Horne, 1983). In this regard, the generation of formaldehyde from ascorbate at elevated temperatures can result in the overestimation of methylated derivatives and underestimation of all other naturally occurring derivatives. To minimize these effects, the extraction buffer developed by Wilson and Horne (1984) was used in the present study. When 10-HCO-H_4PteGlu was chemically synthesized and subsequently subjected to the extraction protocol, no loss of this folate was detected. However, if incubated with pea cotyledon carboxypeptidase at pH 6.3, this formylated compound was largely recovered as 5-HCO-H_4PteGlu (i.e., > 90%). This may be attributed to the instability of the 10-formyl derivative under the conditions employed for hydrolase treatment. Therefore, in neutral to alkali solutions, the intrinsic stability of 5-HCO-H_4PteGlu

over 10-HCO-H_4PteGlu means that the former form of the formyl coenzyme will ultimately accumulate (Figure 13). The use of a pea cotyledon GGH to hydrolyze pteroylpolyglutamates (Roos and Cossins, 1971; Clandinin and Cossins, 1972) involved incubation at pH 4.5, a pH favorable for the production of 5,10-CH^+-H_4PteGlu from both formyl folates (Figure 13). Thus when the conjugase-treated extracts were chromatographed at pH 6.0, 10-HCO-H_4PteGlu and 5-HCO-H_4PteGlu would be generated. Hence, GGH treatment conditions play a critical role in the recovery of certain folates. It should be noted that studies involving the use of pea cotyledon GGH at pH 4.5 produced some folyldiglutamate products (Roos and Cossins, 1971; Clandinin and Cossins, 1972), a phenomenon not observed at the optimal pH for the enzyme.

Horne's group (Wilson and Horne, 1984; Horne *et al*, 1989; Balaghi *et al*, 1993; Horne and Holloway, 1997) employed a GGH activity from rat plasma which displays a pH optimum of 7.0-7.5 (Horne *et al*, 1981b), a pH similar to that of the extracting medium. A chicken pancreas GGH might have been a suitable source of hydrolase in the present studies as its pH optimum of 7.8 closely approximates that of the extraction buffer used. However, this enzyme is less useful when folates are separated by chromatographic methods because it yields a series of pteroyldiglutamate products (Leichter *et al*, 1977). Another commonly used GGH is that from hog kidney. This enzyme would likely be of limited value in the present studies as it is affected by inhibitors found in plant extracts (Olson *et al*, 1948). This

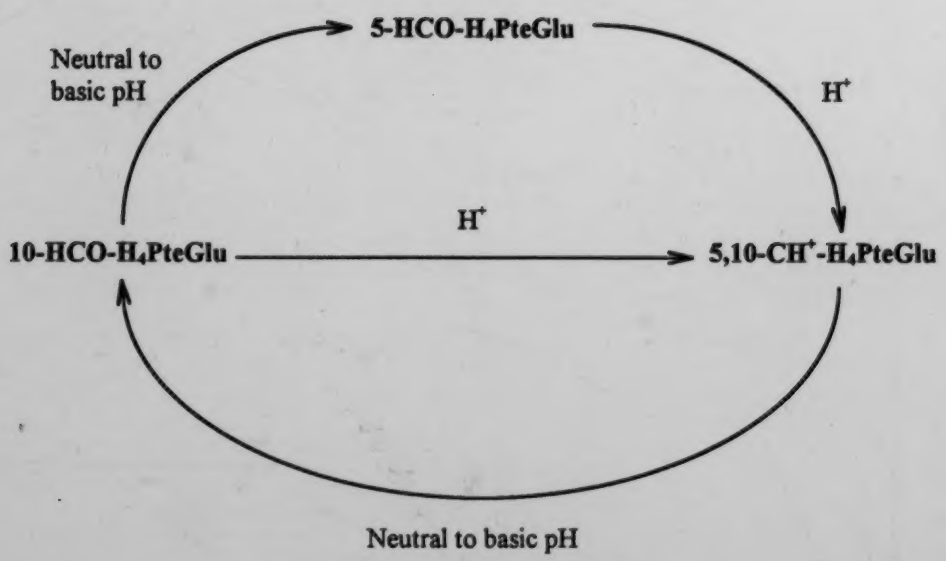


Figure 13. *The pH-dependent Interconversions of Formyl-substituted Folates.* The scheme is derived from Lucock *et al* (1996). Formyl-substituted folate coenzymes are unfortunately the most difficult to quantify owing to the complex pH-dependent interactions involved. In acidic conditions, both 5- and 10-HCO-H_4PteGlu are converted to 5,10-CH^+-H_4PteGlu.

together with a pH optimum of 4.5, like that of a human plasma GGH (Wolff *et al*, 1949), make both sources of conjugase unsuitable for work requiring the assay of individual derivatives. Clearly, the use of rat plasma GGH in the present study could have been useful in quantifying 10-HCO-H_4PteGlu pools. Unfortunately, a reliable source of this protein was not available when these studies were initiated.

4.1.3. HPLC separation of pteroylmonoglutamates

The use of an isocratic ethanol program was capable of fractionating hydrolyzed samples into their individual coenzymes (Figure 8). This program also allowed the ethanol concentration to remain constant in each of the fractions collected thereby eliminating the inhibitory effects ethanol has on the growth of the assay microorganisms. As noted earlier (section 3.4), this HPLC program allowed all of the common folate derivatives to be recovered in the 90-105% range.

4.2. The Folate Content of Pea Leaves

4.2.1. Folate content as a function of leaf age

In agreement with earlier work (Roos and Cossins, 1971), the folate status of pea leaves changed as a function of its age. Thus, 14 d pea leaves contain less folate than 9 and 11 d leaves (Table 3). In related work (Spronk and Cossins, 1972), 30-40 d pea leaves were found to possess even smaller amounts of folate (~ 3.26 nmol g^{-1} fresh weight). Also, 4 week old leaves have been reported to contain approximately

4-fold less folate (1.20 nmol g^{-1} fresh weight) than their 10 d counterparts (Zheng *et al*, 1992). This latter study also revealed a similar trend in leaves of *Lycopersicon esculentum*. Therefore it follows that younger tissues, which have higher metabolic rates centering on nucleic acid, amino acid, and protein synthesis, also have higher levels of folate-dependent one-carbon metabolism. However, these changes are not reflected in the distribution of folate coenzymes of the three extracts examined (Table 3). A study with a longer time frame may prove helpful in determining how these processes affect the folate status of higher plants.

In addition to having a higher total folate content, the younger tissues were also found to have a greater proportion of long-chain folates (Table 4). This was also observed in the radicles of *Pisum sativum* (Sengupta and Cossins, 1971). While monoglutamates and short-chained polyglutamates serve in all folate-dependent reactions, they generally do so less efficiently than longer chained polyglutamates. Conceivably, when folates are present in excess, it may be wasteful of both ATP and glutamate to generate long-chained polyglutamates. With limited levels of folate however, which may be true of the younger, more metabolically active 9 d leaf extracts, polyglutamate formation increases the retention of folate and the folate molecule becomes a more efficient C_1 carrier (Shane, 1995).

4.2.2. Intracellular distribution of folate in 14 d pea leaves

As folate and folate-dependent enzymes are present in mitochondria, chloroplasts, nuclei, and in the cytosol (for review, see Cossins and Chen, 1997), it was of interest in the present study to examine the subcellular distribution of folate derivatives. In mammalian tissues, folate content is relatively more abundant (e.g., 15-30 nmol g^{-1} liver) on basis of fresh weight than in plant tissues and is localized almost exclusively between the cytosol and the mitochondria (Horne *et al*, 1989; Balaghi *et al*, 1993). In contrast, 14 d pea leaves contain significantly smaller amounts of folate (4.72 ± 0.10 nmol g^{-1} fresh weight) and this is primarily sequestered in the cytosol (Table 5). In this compartment, 5-CH_3-H_4PteGlu$_n$ accounts for the majority of the recovered folate. The cytosolic pool also contains 10-HCO-H_4PteGlu$_n$. Since the bulk of SYN, DHY, and CYC activities occur in the cytosol (Chen *et al*, 1997), it follows that this compartment may be a major site for the interconversion of 10-HCO-H_4PteGlu$_n$ and 5,10-CH_2-H_4PteGlu$_n$. This is true of yeast where metabolic flux through GDC, SHMT, and C_1-THF synthase was examined using ^{13}C NMR (Pasternack *et al*, 1992 and 1994b). Although mitochondrial and cytosolic isoenzymes of both SHMT and C_1-THF synthase exist in these cells, approximately 90% of the total utilization of formate for serine synthesis occurs via the cytosolic form of C_1-THF synthase. The inability to detect 5-HCO-H_4PteGlu$_n$ in the cytosolic fraction was surprising in light of its role in mammalian cells (Stover and Schirch, 1992). Due to the relatively high stability of this folate, it is unlikely that it

was oxidized during extraction. The 5-formyl derivative is synthesized from 5,10-CH^+-$H_4PteGlu_n$ by SHMT and metabolized by 5,10-CH^+-$H_4PteGlu_n$ synthetase in animals (see section 1.2.3.1). While neither the 5-HCO-$H_4PteGlu_n$ generating activity of SHMT nor 5,10-CH^+-$H_4PteGlu_n$ synthetase activity have been reported in plant sources, they are believed to be of widespread occurrence. Therefore, the combined activities of SHMT and 5,10-CH^+-$H_4PteGlu_n$ synthetase may be used to buffer 5-HCO-$H_4PteGlu_n$ concentrations (Girgis *et al*, 1997). A high turnover of this folate (Figure 14) may explain its absence in the cytosol. In this regard, mammalian cells have been found to contain very low levels of 5-HCO-$H_4PteGlu_n$ in the cytosol (Horne *et al*, 1989; Balaghi *et al*, 1993; Horne and Holloway, 1997).

A recent study by Neuburger *et al* (1996) used a radioassay procedure to examine the intracellular distribution of folate in pea leaves. These workers detected 0.40 ± 0.05 nmol folate mg^{-1} protein in purified mitochondria, a value similar to that shown in Table 5. They concluded that the mitochondrial compartment accounted for most of the cellular folate. In contrast, only approximately one-tenth of leaf folates was associated with the mitochondrial fraction in the present study. The reason for this discrepancy may include significant losses of cytosolic folate during extract concentration, and the fact that radioassays are generally less sensitive than microbiological assays (Cossins, 1984). It has also been reported that radioassays have limited use in the measurement of folylpolyglutamates (Shane *et al*, 1980). In higher plants, if all cellular folates are synthesized *de novo* in the mitochondria

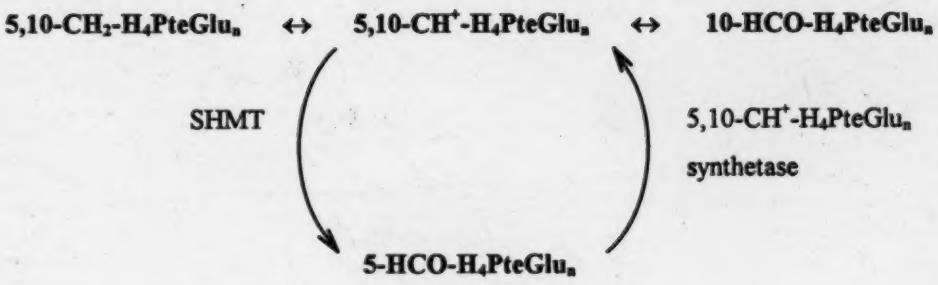


Figure 14. *A Futile Cycle Involving SHMT and 5,10-CH^+-H_4PteGlu$_n$ Synthetase.* Buffering of 5-HCO-H_4PteGlu$_n$ concentrations may involve the combined activities of SHMT and 5,10-CH^+-H_4PteGlu$_n$ synthetase.

(Neuburger *et al*, 1996; Rébeillé *et al*, 1997), it follows that precursors of the larger, cytosolic folate pool must be transported across the mitochondrial membranes. This possibility warrants further study.

While the mitochondrial and chloroplastic folate pools are fairly similar in size (see section 3.5.2), they are very different when expressed on a protein basis. Thus, the mitochondrial pool is approximately 25-fold more concentrated on a protein basis than the chloroplastic pool and is composed of less methylated derivatives than that found in the plastids. The mitochondrial folate pool, contains principally 5-HCO-$H_4PteGlu_n$ and $H_4PteGlu_n$ (Table 5). The latter derivative has importance in the interconversion of glycine and serine in leaf mitochondria (Cossins and Chen, 1997) but the origins and metabolic roles of 5-HCO-$H_4PteGlu_n$ are less clear. In animals, this coenzyme is derived from 5,10-CH^+-$H_4PteGlu_n$ in a reaction catalyzed by SHMT (Stover and Schirch, 1990). The inhibitory effects of the formyl derivative on many bacterial, fungal, and mammalian enzymes (see section 1.2.3.1) has yet to be reported for plant mitochondria. The role of this folate in leaf mitochondria should therefore be elucidated.

The significant loss of 5-CH_3-$H_4PteGlu_n$ seen when stripped thylakoids were chromatographed (see section 3.5.2) suggests that these chloroplastic folates may be present in the stroma. This is supported by the finding that losses of folate in the order of 70% are encountered with repeated washing of these organelles (Shah and Cossins, 1970; Cossins and Shah, 1972). Leaching of folates from chloroplasts led

some investigators to isolate these organelles in non-aqueous media (Rogers *et al*, 1966). While the present study employed aqueous media to isolate chloroplasts (Schuler and Zielinski, 1989), they were found to contain 8-fold more folate on a protein basis than an earlier study (Neuburger *et al*, 1996) which estimated folate content using a radioisotopic binding assay. Despite the apparent efficacy of this method in a clinical setting, a detailed assessment is needed before this procedure can replace standard microbiological methods (Cossins and Chen, 1997). However, the high levels of the 5-CH_3-H_4PteGlu$_n$ found in intact chloroplasts may be required to generate chlorophyll.

Future work in this area should concentrate on studying mitochondrial, chloroplastic, and nuclear folate pools during seed germination and early seedling growth.

4.3. The Effect of Greening on Folate Pools

4.3.1. Folate content of etiolated and greening pea leaves

It has previously been established that light affects the levels of folate derivatives (Spronk and Cossins, 1972). However, prior to the current study, information about the effect of illumination on subcellular folate pools was lacking. Preliminary studies with whole-leaf extracts revealed that folate content increases nearly 2-fold in 9 d etiolated seedlings exposed to 48 h of light (Figure 10). This increase was accompanied by an increase in the specific activity of glycolate oxidase

(see section 3.6.1) which suggests the onset of photorespiration. The possibility that a correlation exists between folate content and metabolic intensity is further suggested by the higher levels of these derivatives in tissues with photosynthetic capacity. The higher levels present in green tissue may also be related to a requirement for C_1 units in the biosynthesis of chlorophyll in addition to involvement in other aspects of chloroplast metabolism. In this regard, the methylated derivative contributed slightly less to the total folate pool after illumination as opposed to before the treatment period (Figure 12). This may be reflective of the use of 5-CH_3-H_4PteGlu$_n$ in *S*-adenosylmethionine biosynthesis (see section 1.2.6). Alternatively, during the development of photorespiration in greening tissues, the activities of GDC and SHMT increase (Turner *et al*, 1993; Vauclare *et al*, 1996). The cleavage of either glycine or serine would result in the formation of 5,10-CH_2-H_4PteGlu$_n$, a derivative which would appear as H_4PteGlu$_n$ in the neutral, aqueous extracts employed for this study. Some of the 5,10-CH_2-H_4PteGlu$_n$ would then be available to be oxidized to 10-HCO-H_4PteGlu$_n$ via DHY and CYC or to 5-HCO-H_4PteGlu$_n$ through DHY and SHMT. It is notable that under these conditions, the specific enzyme activities of DHY and CYC have been reported to increase over 2-fold along with an increment of DHY-CYC-Ab cross-reacting protein (Chen *et al*, 1997).

While the quantity of folate increased dramatically over the 72 h greening period, these newly synthesized derivatives possessed relatively short glutamate chains (Figure 11). The slow rate at which polyglutamate formation occurs has been

well documented (Shane, 1989). Therefore, as more monoglutamate substrates for FPGS become available, a smaller proportion of long-chain folates will accumulate. As folates are only retained within organelles and cells if they are charged with three or more glutamate residues (Shane, 1990), this may represent a period in which folates synthesized in the mitochondria (Neuburger *et al*, 1996; Rébeillé *et al*, 1997), may be transported into the cytosol. Interestingly, DHY and CYC activities from pea cotyledons do not show a preference for polyglutamate substrates (Kirk *et al*, 1995).

4.3.2. Changes in the intracellular distribution of folate during greening

Two mitochondrial folate-dependent enzyme activities, GDC and SHMT, increase with the development of photorespiration in greening tissues (Turner *et al*, 1993; Vauclare *et al*, 1996). As GDC catalyzes the mitochondrial production of 5,10-CH_2-H_4PteGlu$_n$, greening will provide more substrate for mitochondrial DHY and SHMT. In this regard, mitochondrial folate content increased 10-fold and on a protein basis, was 3-fold more concentrated (Tables 7-9). In addition, greening decreased the proportion of methylated derivatives found in mitochondria of etiolated tissues (see section 3.6.2), probably due to increases in the unsubstituted and 5-HCO-H_4PteGlu$_n$ pools. It is likely that most of the 5,10-CH_2-H_4PteGlu$_n$ will be utilized in serine biosynthesis as the specific activity of SHMT is approximately 15-fold higher than DHY in pea leaf mitochondria (Neuburger *et al*, 1996). However, generation of

serine from 5,10-CH_2-H_4PteGlu$_n$ is probably not the only origin of this amino acid in leaf tissue as serine can also be synthesized via glycolysis from phosphoserine (Wagner, 1995). Therefore, the metabolic role of mitochondrial DHY and CYC may be to convert 5,10-CH_2-H_4PteGlu$_n$ to 10-HCO-H_4PteGlu$_n$, which is required for the synthesis of formylmethionyl-tRNA, the initiator of protein synthesis in these organelles (Staben and Rabinowitz, 1984; Coffin and Cossins, 1986). The relatively low levels of mitochondrial SYN activity after greening (Chen *et al*, 1997) suggest that the activation of formate is probably a minor route for 10-HCO-H_4PteGlu$_n$ biosynthesis in mitochondria of photosynthetic tissues. Therefore, pea mitochondria probably generate this folate by DHY and CYC activities during photorespiration.

Serine is exported by mitochondria and converted to glycerate by peroxisomal enzymes during photorespiration (Douce and Neuburger, 1989). As the chloroplastic and cytosolic compartments of leaves contain SHMT (Neuburger *et al*, 1996), some of this exported serine may be available for the biosynthesis of 5,10-CH_2-H_4PteGlu$_n$. In other eukaryotes, the cytosolic pool of this folate has importance in the formation of purines, thymidylate, methionine, and choline (Schirch, 1984). While relatively little is known about the nature and compartmentation of purine biosynthesis in plants (Cossins and Chen, 1997), it is conceivable that green tissues generate the 10-HCO-H_4PteGlu$_n$ needed in this pathway by cytosolic and/or chloroplastic DHY and CYC activities. Thus during photorespiration, some of the serine formed in the mitochondria may enter either pathway to generate C_1-substituted folates for other

pathways of one-carbon metabolism. Future studies on the folate pool in plastids of greening tissues would prove interesting as these organelles contain folate derivatives and folate-dependent enzymes (Cossins, 1980).

4.4. Conclusions

The use of glycerol-cryoprotected bacteria, 96-well microtiter plates, and ion-pair reverse phase HPLC, to study folate metabolism has been well documented (Horne *et al*, 1989; Balaghi *et al*, 1993; Horne and Holloway, 1997). The present study successfully applied these methods to determine the intracellular distribution of folate in pea leaves. If in fact the biosynthesis of polyglutamyl folate is mainly localized in the mitochondria (Neuburger *et al*, 1996; Rébeillé *et al*, 1997), the majority of cellular folate must be transported across the mitochondrial membranes as the bulk of tetrahydrofolates in pea leaves are extra-mitochondrial. Because the mitochondrial pool of folate in pea leaves is dominated by tetraglutamate (25%) and pentaglutamate (55%) derivatives (Besson *et al*, 1993), the free diffusion of this coenzyme within the cell is very limited (Shane, 1990). Therefore, the mode by which folate is transported between the different compartments remains to be answered. Furthermore, *p*-ABA, which is required for the biosynthesis of folate (see section 1.1.1), is synthesized from chorismic acid through the shikimic acid pathway. As this pathway is localized in the plastids (Herrmann, 1995), the transport of *p*-ABA to the mitochondria also requires further work. The folate pools of the three compartments

examined also differed in concentration and one-carbon substitution. This clearly demonstrates the distinct pools of folate which exist in pea leaf cells and is reflective of the different roles that this coenzyme may play in the various compartments.

The changes observed in the pteroylglutamate content of pea leaves with aging and with the onset of photorespiration are reflective of the need for DNA and protein synthesis. Hence, more metabolically active plants have an increased demand for one-carbon units and a greater folate content to meet this requirement. In the leaves of green plants, the mitochondrial GDC and SHMT reactions act in the photorespiratory pathway to recycle carbon produced as a result of the oxygenation of ribulose 1,5-bisphosphate. However, as the maximal velocity of the GDC is approximately 2.5-fold higher than the maximal rate of serine synthesis through SHMT (Rebeille *et al*, 1994), some of the 5,10-CH_2-H_4PteGlu$_n$ generated by GDC may be available for other pathways of folate metabolism. In this regard, the mitochondrial folate pool increased 10-fold and changed in composition from mainly methylated derivatives to formylated and unsubstituted folates when etiolated leaves were greened for 48 h. As the plastids also contain folate derivatives and folate-dependent enzymes, it is possible that changes in the folate content of these organelles may also accompany the onset of photorespiration.

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